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LXXVII. EXPERIMENTS ON NUTRITION.
X. COMPARATIVE VITAMIN B_1 VALUES OF FOODSTUFFS. CEREALS II.

By ROBERT HENRY ADERS PLIMMER,
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(*Received March 23rd, 1931.*)

In our previous experiments [Plimmer, Rosedale, Raymond and Lowndes, 1927] on testing cereals for their vitamin B (B_1) value, rearing of young by pairs of adult pigeons was taken as the standard of comparison. Some data concerning the maintenance of adult birds were also then given. A greater amount of foodstuff containing vitamin B was needed for rearing than for maintenance. This fact has been again observed in subsequent experiments.

It was considered at that time that the standard of rearing was too high and not altogether satisfactory. Other factors might well be concerned in the hatching of eggs and rearing of young, such as the presence of fat-soluble vitamins which were not allowed for. The standard of maintenance for a period of at least 26 weeks was adopted in our trials with pulses and nuts [Plimmer *et al.*, 1929] with satisfactory results. In order to bring cereals into proper comparison it was necessary to test them again on this standard. The results of these experiments are herewith presented.

In addition we have made some experiments on the extraction of vitamin B from wheat germ and have again tested marmite for comparison with the cereals and for ascertaining if its value were the same after a period of 5 or 6 years.

The term vitamin B is now used to include several unknown factors. In these tests the symptoms of head retraction of pigeons have been taken as the sign of insufficient vitamin B, *i.e.* in the modern nomenclature vitamin B_1.

Experimental.

The experiments have been made with pairs of pigeons kept in cages on the roof of the building as in previous trials with pulses.

The food was made into pills of white flour and the test grain which was ground finely in a coffee mill. In the earlier experiments cod-liver oil was not included, but later 1 % was added so that the birds should not suffer from a shortage of fat-soluble vitamins. Fishmeal was included to supply protein and mineral salts.

The results are recorded in a series of tables in the same form as in our earlier experiments with pulses.

Wheat.

In our previous paper it was reported that a pair of adult pigeons had been maintained on a diet containing 50 % whole wheat flour for 45 weeks, and that another pair (Nos. 93 and 26/27) had been kept on 40 % whole wheat flour for 23 weeks. This pair has been continued on the same diet for 78 weeks. They lost a little weight in the first 20 weeks, but at 40 weeks were above their original weight. Then followed a small decline and after 78 weeks they were slightly below their original weight. The hen laid some eggs at the start, but not again till the 46th week. On reducing the quantity of whole wheat to 30 % both birds lost weight, became ill, showed polyneuritis and died. 40 % wheat was clearly the minimum for maintenance (Table I).

Table I. *Whole wheat.*

Date	Time in weeks	Diet			Weights, g.		Remarks
		Whole wheat	White flour	Fish-meal	Cock No. 93	Hen No. 26/27	
30. iii. 27 to 17. viii. 27	20	40	55	5	385	295	Six eggs. One squab hatched and reared, but poor growth. Other eggs deserted
4. i. 28 to 23. v. 28	60				440	355	
26. ix. 28 to 24. x. 28	4	30	65	5	335	305	Twelve eggs; all deserted. Both showed retracted head positions and died
4. i. 28 to 26. ix. 28	78				400	330	
30. iii. 27 to 4. i. 28	40				530	430	

Bran.

Two pigeons had previously been maintained on 30 % bran for 32 weeks. The experiment has been repeated and young have been reared on this quantity. The same pigeons were then maintained on 20 % bran and a pair of young was reared. On reducing the quantity to 10 % both birds lost weight and showed polyneuritis. 10 % bran was insufficient as verified by testing with another pair of birds (Table II).

Middlings.

Previously, a pair of pigeons had been maintained on a diet containing 30 % of middlings mixed with white rice for 16 weeks. In these experiments some of the middlings on account of the small particles could not be picked up so that white flour pills were substituted; a longer time for maintenance was also essential. The trial was started with a pair of birds on 30 % middlings which was reduced to 20 % after a short period on whole wheat. Cod-liver oil (1 %) was now mixed with the food to ensure the presence of fat-soluble vitamins. They were maintained on this diet for 27 weeks and again after an interval on whole wheat the amount of middlings was reduced

Table II. *Bran.*

Date	Time in weeks	Diet: Bran	White flour	Fish-meal	Cod-liver oil	Weights, g.: Cock No. 75	Hen No. 76	Remarks
8. v. 29						510	360	Eight eggs laid, four hatched
to		30	65	5	0			and young reared. Four
13. xi. 29	27					585	450	hatched and young died
to			On whole wheat					
11. xii. 29	4					595	410	Ten eggs laid; first two
to		20	74	5	1			hatched and young reared, Nos. 4 and 6 below. Eight
18. vi. 30	27					535	405	deserted
to		10	84	5	1			
2. vii. 30	2					455	355	Cock showed head retraction and died
to								
23. vii. 30	5					—	305	Hen showed head retraction and died
						No. 4	No. 6	
29. i. 30						95	105	Squabs reared on 20 % bran
to		20	74	5	1			
6. viii. 30	28					460	425	
						No. 9	No. 29	
28. v. 30						425	430	
to		10	84	5	1			
9. vii. 30	6					245	310	Cock showed paralysis and died
to								
23. vii. 30	8					—	245	Hen showed head retraction and died

to 10 %. They were quite well on this quantity, but on reducing the amount to 5 % they lost weight and showed the symptoms of polyneuritis.

The experiment was confirmed with another pair of pigeons, starting at 10 % middlings. The insufficiency of 5 % middlings was verified with a third pair of pigeons. The details are shown in Table III. The sample of middlings would thus appear to contain a considerable proportion of wheat germ, the value of which we had found to be 6 % for maintenance. Other samples of middlings may not have such a high vitamin B value. We have not tested any other samples.

Wheat germ.

For comparison with whole wheat, middlings and bran some further trials have been made with wheat germ. No difference was previously noticed between fresh and heated wheat germ. In these experiments "bemax" was used as source of wheat germ. Maintenance was secured on 6 %, but on prolonging the test both birds died without showing the typical signs of polyneuritis. Two pairs of birds were maintained on 7 % for 26 weeks and one pair was kept for 53 weeks. The details are shown in Table IV. The material had thus the same value as the wheat germ of the previous trial.

Extraction of wheat germ with solvents. With a view of extracting and concentrating vitamin B_1 some experiments were first made to ascertain the completeness of the extraction with solvents. Fat solvents, such as ether, chloroform, light petroleum do not extract vitamin B_1. The residue after

Table III. *Middlings.*

Date	Time in weeks	Diet: Middlings	Diet: White flour	Diet: Fish-meal	Diet: Cod-liver oil	Weights, g.: Cock No. 15	Weights, g.: Hen No. 44	Remarks
8. v. 29						370	375	
to		30	65	5	0			Seven eggs. First two hatched and young reared; one died later
13. xi. 29	27					455	445	Other eggs deserted
to		On whole wheat						No eggs
11. xii. 29	4					410	435	
to		20	75	5	0			No eggs
12. iii. 30	13					415	450	
to		20	74	5	1			Nine eggs. One hatched, squab died. Others deserted
18. vi. 30	14					430	425	
to		10	84	5	1			Six eggs at beginning, none later
17. xii. 30	26					455	420	
to		5	89	5	1			
14. i. 31	4					335	335	Hen showed head retraction and was cured. Cock had paralysis and died
						No. 85	No. 86	
28. v. 30						460	420	
to		10	84	5	1			Six eggs; all deserted
31. xii. 30	31					550	435	
to		5	89	5	1			
28. i. 31	4					390	320	Cock showed head retraction and was cured. Hen had head retraction and died
						No. 100	No. 1557	
5. xi. 30						420	375	
to		5	89	5	1			
10. xii. 30	5					300	315	Both showed head retraction and were cured

Table IV. *Wheat germ.*

Date	Time in weeks	Diet: "Bemax"	Diet: White flour	Diet: Fish-meal	Weights, g.: Cock No. 329	Weights, g.: Hen No. 327	Remarks
28. ix. 27					330	330	
to		6	89	5			Six eggs; deserted
18. iv. 28	29				355	340	Cock died
to							
27. vi. 28	39						Hen died. Both birds at *p.m.* examination had very wet tissues and very large hearts
					No. 88	No. 80	
1. viii. 28					380	410	
to		7	88	5			No eggs during winter months
20. ii. 29	29				465	495	
					No. 89	No. 49	
26. ix. 28					390	345	
to							Four eggs; deserted
20. iii. 29	25				450	385	
to							
16. x. 29	30				410	365	

extraction, as shown in Table IV *a*, fed to pigeons in an amount of 6 % gave maintenance for 26 weeks. Extraction with absolute alcohol subsequent to

Table IV *a*.

Date,	Time in weeks	Diet: Ether-extracted "bemax"	Diet: White flour	Diet: Fish-meal	Weights, g.: Cock No. 54	Weights, g.: Hen No. 75	Remarks
28. ix. 27					385	400	
to		6	89	5			
24. iv. 28	30				365	350	
to							
26. ix. 28	52				350	320	Two eggs in 43rd week; deserted
		Ether + abs. alc.-extracted "bemax"			No. 64	No. 520	
8. ii. 28					465	495	
to		10	85	5			Eighteen eggs; deserted
8. viii. 28	26				440	470	
to			On wheat				Two eggs; deserted
29. viii. 28	3				420	455	
to		6	89	5			
17. x. 28	7				350	390	Two eggs; deserted. Cock had head retraction. Cured
to							
31. x. 28	9				—	305	Hen died. Had large heart
		Light petroleum + abs. alc.-extracted "bemax"			No. 88	No. 80	
20. ii. 29					465	495	
to		7	88	5			Twelve eggs; deserted
21. viii. 29	26				435	455	
		Abs. alc.-extracted "bemax"			No. 26/28	No. 58	
13. ii. 29					445	360	
to		50	47·5	2·5			
7. viii. 29	25				395	330	No eggs
		Petroleum + 50 % alc.-extracted "bemax"			No. 93	No. 50	
20. iii. 29					420	470	
to		7	88	5			
24. iv. 29	5				275	305	Both had head retraction
					No. 26/28	No. 58	
4. ix. 29					430	335	
to		21	74	5			
9. x. 29	5				315	275	Both had head retraction
					No. 89	No. 49	
20. xi. 29					450	385	
to		42	53	5			
11. xii. 29	3				350	300	Both had head retraction

these solvents removed only a small quantity of vitamin B_1. Pigeons were maintained with 10 % of the residue in the diet, but failed on 6 %. Complete extraction was effected with 50 % alcohol. The residue was tested in amounts of 7, 21 and 42 % of the diet. The birds showed symptoms of polyneuritis at each level in 3 to 5 weeks. The vitamin was present in the 50 % alcoholic

extract. It is thus not necessary to use acid alcohol for the extraction. Further experiments with the alcoholic extract have been discontinued as similar work is being carried out by Guha and Drummond [1929].

Barley.

55 % of whole barley in the diet with white rice was the smallest amount used previously and the pigeons were maintained for 42 weeks. Two pigeons were started on 40 % barley with white flour to compare with wheat. After 7 weeks they were found to be two cocks and a hen was substituted for one of them. They were maintained for 35 and 27 weeks respectively. After an interval on whole wheat, the barley was reduced to 30 %. Both suffered from polyneuritis. The vitamin B_1 value of barley was thus the same as that of wheat.

Table V. *Barley.*

Date	Time in weeks	Diet				Weights, g.		Remarks
		Barley	White flour	Fish-meal	Cod-liver oil	Cock No. 333	Hen No. 54	
5. ix. 28 to 7. xi. 28	11	40	55	5	0	405 420	— 345	
to 15. v. 29	29					415	355	Four eggs; one hatched, squab died
to 3. vii. 29	7		On whole wheat					Two eggs; one hatched and reared
to 14. viii. 29	6	30	65	5	0	320	325	Four eggs; deserted Cock had head retraction and died
to 11. ix. 29	10					—	210	Hen had head retraction and died

Malt extract.

Cooper [1914] examined several malt extracts for vitamin B by the curative method and found that two samples had curative action. Stammers [1924] and Southgate [1924] have also found vitamin B in malt. Randoin and Lecoq [1927] on substituting malt extract for dextrin to the extent of 66 % of the diet obtained cure and prevention of polyneuritis. Bacharach and Allchorne [1928] considered malt extract a rich source of vitamin B and of almost equal value to the cereal from which it was made.

Malt extract had not been previously tested by us. With the pill method of feeding pigeons it was easy to incorporate the sticky material with white flour and try its value in comparison with cereals. The extract tested was the same as is used for patients in St Thomas's Hospital. Two trials were made with 40 % malt in the diet, three with 30 % and one with 20 %. The birds suffered from polyneuritis on 20 %, but were maintained on 30 % and 40 % for 23 and 27 weeks. The value of the malt extract was thus slightly greater than that of barley. The data are given in Table VI.

Table VI. *Malt extract.*

Date	Time in weeks	Diet: Malt extract	Diet: White flour	Diet: Fish-meal	Diet: Cod-liver oil	Weights, g.: Cock No. 24	Weights, g.: Hen No. 70	Remarks
29. v. 29 to 11. ix. 29	15	40	55	5	0	375 260	375 295	Birds ate very little and lost weight. Both not well. Cock had head retraction and was cured. Both got better. No eggs
19. ii. 30 to 13. viii. 30	25	30	65	5	0	415 345	410 305	Hen paralysed and died
to 27. viii. 30	27					310	—	Cock had head retraction and died
						No. 343	No. 60	
23. x. 29 to 19. iii. 30	21	40	55	5	0	465 440	410 375	Ten eggs; all deserted
to 7. v. 30	7	40	54	5	1	425	365	
to 23. vii. 30	11	30	64	5	1	410	320	Four eggs; deserted
to 17. ix. 30	19					390	305	Cock had head retraction and died. Hen was paralysed and died
						No. 98	No. 62	
6. xi. 29 to 8. i. 30	9	30	64	5	1	490 415	405 375	Cock had head retraction and died
to 26. iii. 30	20					—	330	Hen had head retraction and died
						No. 42	No. 38	
19. iii. 30 to 21. v. 30	9	20	74	5	1	455 340	300 245	Both showed head retraction and were cured

Oatmeal and oats.

Previous work led to the conclusion that rearing was not possible on oatmeal without an addition of marmite, though adult pigeons could be maintained for 40 and 52 weeks on 95 % oatmeal. Maintenance experiments with oatmeal were therefore essential for proper comparison with other cereals. Maintenance was found on a diet containing 50 % oatmeal for 31 weeks, and also with 40 % oatmeal. This result was confirmed with a second pair of birds. On reducing the amount of oatmeal to 30 %, the cock bird showed polyneuritis in 28 weeks. 30 % oatmeal was tested again with another pair of pigeons. In this case the hen showed polyneuritis, but not the cock. There was failure on 20 % oatmeal. Maintenance was thus possible on 30 % oatmeal as the minimum quantity, but it is not certain and we ought to take 35 % as the proper minimum quantity. Oats had the same value as oatmeal. The data are given in Table VII.

Table VII. *Oatmeal.*

Date	Time in weeks	Diet: Oat-meal	Diet: White flour	Diet: Fish-meal	Diet: Cod-liver oil	Weights, g.: Cock No. 56	Weights, g.: Hen No. 14	Remarks
29. ii. 28						415	375	
to		50	45	5	0			Twelve eggs. Ten deserted. Hatches from last two,
3. x. 28	31					400	390	but squabs died
						No. 57	No. 6	
1. viii. 28						465	385	
to		40	55	5	0			Two eggs at beginning, hatched but squabs died
27. ii. 29	29					540	515	No more eggs
						No. 236	No. 99	
1. v. 29						475	390	
to		40	55	5	0			Ten eggs. Four hatched and reared with poor growth and weak. Two hatched
13. ii. 29	28					525	445	and died. Four deserted
to								
8. i. 30			On whole wheat			555	405	
to		30	64	5	1			Ten eggs. Two hatched; squabs died. Others deserted
23. vii. 30	28					425	445	Cock showed head retraction and was cured
to								
6. viii. 30	30	(Oats)				475	415	
to		30	64	5	1			Four eggs; deserted.
1. x. 30	8					380	400	Cock showed head retraction and was cured. Hen
to								kept on diet
11. ii. 31	27					—	410	
						No. 1156	No. 35	
16. vii. 30						410	400	
to		30	64	5	1			Two eggs; deserted
29. x. 30	15					410	330	Hen paralysed and died.
to								Cock continued on diet
11. ii. 31	15					420	—	
						No. 56	No. 38	
30. vii. 30						395	305	
to		20	74	5	1			
17. ix. 30	7					285	215	Both showed head retraction. Hen died; cock cured

Buckwheat.

Buckwheat had not been found a satisfactory grain for rearing, but a pair of pigeons had been maintained on 70 % buckwheat in the diet for 31 weeks. Proper comparison with other cereals was needed. A pair of pigeons was kept for 29 weeks first on 40 %, then on 30 % and later on 20 %. On reducing the amount of buckwheat to 10 %, both birds showed polyneuritis and died. The data are in Table VIII. Buckwheat is thus slightly richer in vitamin B than the other grains.

Table VIII. *Buckwheat.*

Date	Time in weeks	Diet: Buck-wheat	White flour	Fish-meal	Cod-liver oil	Weights, g.: Cock No. 334	Hen No. 335	Remarks
10. x. 28						375	345	
to		40	55	5	—			Five eggs; deserted
1. v. 29	29					360	340	
to			On whole wheat					Two eggs; deserted
29. v. 29	4					350	335	
to		30	65	5	—			Seven eggs; deserted
18. xii. 29	29					410	400	
to		20	74	5	1			Eight eggs; deserted
6. viii. 30	29					365	340	
to			On whole wheat					One egg; deserted
17. ix. 30	6					345	320	
to		10	84	5	1			Two eggs; deserted
12. xi. 30	8					255	280	Cock had head retraction
to								and died
26. xi. 30	10					—	255	Hen showed paralysis and died

Maize.

The experiment with maize carried out previously was maintenance on 60 % maize with 35 % white flour for 18 weeks. The same pair of pigeons after an interval on whole wheat was tested with 40 % maize. As seen from Table IX they showed polyneuritis in about 20 weeks. This amount was thus insufficient. No further experiment was made, as previously a pair had been maintained on 50 % maize for 23 weeks.

Table IX. *Maize.*

Date	Time in weeks	Diet: Maize	White flour	Fish-meal	Weights, g.: Cock No. 26/4	Hen No. 26/5	Remarks
5. x. 27					445	395	
to		40	55	5			Ten eggs; deserted
15. ii. 28	19				380	290	Hen had head retraction
to							and died
22. ii. 28	20				360	—	Cock had head retraction and died

Rye.

With rye it had been observed that a pair of birds could be maintained for 31 weeks on a diet containing 45 %. The next trial was to use 40 %. The first cock bird was not satisfactory, but a second was kept on this amount for 26 weeks and the hen for a total of 47 weeks. On reducing the amount of rye to 30 %, both birds showed polyneuritis. This was confirmed with another pair and another hen put in with the cock after the first hen had died. The cock bird seemed rather resistant to shortage of vitamin B and only showed symptoms after 39 weeks. During the last period of 6 weeks his food consumption was measured and was from 10 to 13 g. daily as compared with a normal consumption by healthy birds of 30 to 40 g. daily. On a small consumption of food it has often been noticed that pigeons will live for long

periods before they show head retraction, or die showing only symptoms of paralysis. The data are in Table X.

Table X. *Rye.*

Date	Time in weeks	Diet: Rye	Diet: White flour	Diet: Fish-meal	Weights, g.: Cock No. 77	Weights, g.: Hen No. 25/27	Remarks
27. vi. 28					405	360	
to		40	55	5			
12. ix. 28	11				330	365	Cock changed
					No. 87		
					395	—	
to							Four eggs; deserted
14. xi. 28	10				475	385	Cock escaped
					No. 64		
					420	—	
to							Fourteen eggs; deserted
22. v. 29	26	(47)			425	345	
to		On whole wheat					
19. vi. 29					420	380	
to		30	65	5			Two eggs; deserted
14. viii. 29	8				340	275	Cock had head retraction and died
to							
28. viii. 29	10				—	255	Hen had head retraction and died
					No. 167	No. 82	
5. ix. 28					415	320	
to		30	65	5			
28. xi. 28	12				340	250	Hen had head retraction and died
						No. 94	
9. i. 29	18				440	385	
to							
1. v. 29	13				320	285	Hen had head retraction and was cured
to							
5. vi. 29	39				285	—	Cock had head retraction and was cured

Dari and millet.

Rearing experiments only were made previously with these grains. The smallest amounts with white rice were 55 %. Tests have now been made using white flour pills with 40 % of the grains. On this amount the pigeons

Table XI. *Dari.*

Date	Time in weeks	Diet: Dari	Diet: White flour	Diet: Fish-meal	Diet: Cod-liver oil	Weights, g.: Cock No. 84	Weights, g.: Hen No. 73	Remarks
7. xi. 28						535	355	
to		40	55	5	0			Four eggs; one hatched, squab died
15. v. 29	27					515	385	
to		On whole wheat						Four eggs; one hatched and squab reared
19. vi. 29	5					560	370	
to		30	65	5	0			
20. xi. 29	22					435	320	Both birds had head retraction and were cured

were maintained with both grains. They failed on 30 % dari, and on 20 % millet. The data are in Tables XI and XII.

Table XII. *Millet.*

Date	Time in weeks	Diet: Millet	White flour	Fish-meal	Cod-liver oil	Weights, g.: Cock No. 100	Hen No. 1557	Remarks
6. iii. 29						395	360	
to		40	55	5	0			Twelve eggs; three hatched,
9. x. 29	31					410	360	one squab reared, two died
to			On whole wheat					Two eggs; deserted
6. xi. 29	4					445	380	
to		30	64	5	1			Eight eggs; one hatched,
4. vi. 30	30					415	375	squab died, others deserted
to			On whole wheat					Two eggs; hatched and
23. vii. 30	7					410	350	squabs reared
to		20	74	5	1			
3. ix. 30	6					270	305	Cock had head retraction
to								and was cured
10. ix. 30	7					280	270	Hen had head retraction and was cured

Rice.

It was of interest and importance to compare the value of brown rice, *i.e.* rice with its pericarp, with that of other cereals. Tests were started with 40 % rice. On this amount the pigeons were maintained, but they suffered from polyneuritis on 30 %. Such rice has thus the same value in vitamin B as wheat and other cereals. The results are in Table XIII.

Table XIII. *Rice.*

Date	Time in weeks	Diet: Brown rice	White flour	Fish-meal	Cod-liver oil	Weights, g.: Cock No. 88	Hen No. 80	Remarks
25. ix. 29						445	455	
to		40	55	5	0			Six eggs; deserted
16. iv. 30	29					430	475	
to		30	64	5	1			
28. v. 30	6					370	420	Hen died, new hen used
to							No. 95	
4 vi. 30						395	365	
to								
25. vi. 30	10					275	350	Cock had head retraction
to								and died
3. ix. 30	13					—	285	Hen had head retraction and died

Marmite.

Partly from our experiments and partly from experiments of other workers the belief has arisen that marmite has not such a high content of vitamin B as it had 8 or 9 years ago. Our first trials [Plimmer, Rosedale, and Raymond, 1923] indicated that 3 % marmite was sufficient to keep a pair of pigeons in health. These trials were of short duration, from 5 to 6 weeks, and are not strictly comparable with those of our present standard of maintenance for at

least 26 weeks. Also, white rice was used instead of white flour, which allowed the birds to reject any grains not properly coated with marmite. It is therefore possible that this amount would not have sufficed in a long trial. Later [Plimmer, Rosedale and Raymond, 1927, pp. 929–931] it was found that amounts varying from 4 to 8 % of different yeast extracts, again tested for short periods, sufficed. The first trial with marmite with long maintenance as the standard indicated that 8 % was the minimum amount.

Since marmite is so often used as the source of vitamin B in experimental diets, it was considered of distinct importance to make tests with marmite of recent manufacture. It was not possible to test marmite manufactured 10 years ago so as to obtain an exact comparison. Two samples have been kindly supplied by the makers, prepared from the same yeast. The first

Table XIV. *Marmite.*

		Diet				Weights, g.		
Date	Time in weeks	Marmite. Ct. marmite	White flour	Fish-meal	Cod-liver oil	Cock No. 89	Hen No. 49	Remarks
15. i. 30						455	380	
to		8	87	5	0			
26. iii. 30	10					455	305	Hen died from burst egg in oviduct
							No. 43	
2. iv. 30	11					460	445	
to		8	86	5	1			Eight eggs; one hatched,
16. vii. 30	26, 15					435	460	squab died. Others deserted
to		6	88	5	1			Two eggs, one hatched,
11. ii. 31	30					430	445	squab died
to		4	90	5	1			
25. ii. 31	2					305	375	Both birds had head retraction. Cock cured, hen died
						No. 96	No. 2	
1. x. 30						370	295	
to		4	90	5	1			
19. xi. 30	8					340	315	Cock had head retraction
to								and died
28. i. 31	17					—	—	Hen paralysed and died
		K. marmite				No. 18	No. 39	
15. i. 30						530	505	
to		8	87	5	0			Four eggs; deserted
26. iii. 30	10					515	510	
to		8	86	5	1			Eight eggs; deserted
16. vii. 30	16					475	455	
to		6	88	5	1			Six eggs; deserted
11. ii. 31	30					520	500	
to		4	90	5	1			
4. iii. 31	3					470	400	Hen had head retraction
to								and cured
18. iii. 31	5					350	—	Cock had head retraction and cured
						No. 23	No. 3	
1. x. 30						355	325	
to		4	90	5	1			
19. xi. 30	8					285	275	Cock had head retraction
to								and cured
28. i. 31	17					—	235	Hen paralysed and died

(Ct. marmite) was similar to the material of the last few years, the second (K. marmite) was material made from a clarified extract. This is the present method of manufacture. As may be seen from Table XIV both the samples had the same value. 6 % in the diet was the minimum for maintenance. This compares with 8 % found in 1925. There is thus no essential change in the vitamin B value of marmite in a period of 6 years. It may be noted that marmite if kept for such long periods may lose value, as we observed in 1923. Freshly manufactured marmite seems of approximately the same value from year to year.

Baker's yeast.

The amount of baker's yeast required to supply vitamin B was found previously to be above 10 % of the diet. An experiment with 12 % in the diet sufficed for maintenance of two pigeons for 31 weeks. This value corresponds with its moisture content and reckoned on dry weight its value is the same as that of dried brewer's yeast, 4 % of the diet.

Discussion.

These experiments do not differentiate between the several factors of the vitamin B complex. They give essentially the vitamin B_1 or antineuritic value. The vitamin B_2 value of cereals has been examined by Aykroyd and Roscoe [1929] and has been found to be very small. It was presumably supplied in the fishmeal of our diets and is probably not needed by pigeons according to the work of Carter, Kinnersley and Peters [1930]. If vitamin B_3 is to be regarded as necessary for the maintenance of weight, its amount in cereals and pulses must be in close correspondence with the amount of vitamin B_1. Reference to the weights of the pigeons in our tables shows that weight is maintained over the long periods of 26 or more weeks and loss of weight occurs as soon as the amount of cereal is reduced below the maintenance quantity, followed in a few weeks by the symptoms of head retraction in most cases and paralysis in the others. There was also invariably a fall in food consumption which really accounts for the loss in weight. Occasionally a bird has lived for many weeks at a much lower weight level and with a very small food consumption. The general metabolism of these birds is less and a smaller amount of vitamin B_1 or B_3 or both is then required. The vitamin B complex is wanted for the health of the bird to replenish the store in every cell of the body. At the low weight level the bird eventually dies unless cured, but, if cured, there is a large food consumption and gain in weight. *Post mortem* examination of our dead birds has almost invariably shown that the hearts are very large and flabby, and frequently the tissues of the birds are wet. Maintenance of weight may be due to such a wet condition of the tissues, and should be distinguished from increase of tissue substance.

SUMMARY.

Adopting the same standard of comparison as in our work on pulses and nuts, the comparative vitamin B values of cereals are as follows:

	Percentage amount in diet for maintenance	Comparative vitamin B value
Dried yeast	4	100
Marmite	6	67
Wheat germ ("bemax")	6–7	62
Middlings	10	40
Baker's yeast	12	33
Bran	20	20
Buckwheat	20	20
Millet	30	13
Oatmeal	35	11
Wheat	40	10
Barley	40	10
Malt	40	10
Rye	40	10
Dari	40	10
Brown rice	40	10

Cereals are thus not quite so rich in vitamin B as pulses, which had a comparative figure of 13.

We desire to express our thanks for grants from the Government Grant Committee of the Royal Society and from The Imperial Chemical Industries, Limited, which have defrayed the costs of the foods and birds used in these experiments.

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LXXVIII. STRUCTURE AND ENZYME REACTIONS.

IX. THE SYSTEMS AMYLASE-STARCH-GELATIN AND UREASE-UREA-GELATIN.

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THE previous parts of this series were devoted to the study of the influence of adsorption on the velocity of enzymic reactions in heterogeneous systems. In the present paper experiments on the influence of the state of the sorbent, of its viscosity, occlusion and aggregation (flocculation) will be described.

Increase in the viscosity of the medium in which an enzymic reaction is proceeding in a heterogeneous system should lead to retardation of the velocity of reaction in two ways, by reduction of the number of collisions taking place in unit time between the free enzyme and substrate, and between the enzyme and substrate adsorbed on different colloidal particles. The effect of increasing viscosity was studied in the systems amylase-starch-gelatin and urease-urea-gelatin, differing in that the first system contains a colloidal, slowly diffusing substrate, whilst the substrate of the second system is a rapidly diffusing crystalloid. The phenomenon of occlusion in relation to the velocity of reaction was next studied in the above systems. As the state of aggregation of gelatin increases, monons, together with adsorbed enzyme and substrate particles, cohere, yielding polyons, which may then further aggregate, finally to yield either a coagulum or a jelly. As a result of this process the enzyme or substrate or both may be enclosed within the aggregates, and, should the structure of these be such as to exclude entry of larger particles, may be excluded from reaction. The system amylase-glycogen-caseinogen, described in the preceding part of this series [Przyłęcki and Gurfinkel, 1930] affords an example of occlusion with consequent inhibition of reaction.

EXPERIMENTAL.

I. THE SYSTEM AMYLASE-STARCH-GELATIN.

Starch solution was prepared from starch paste (Merck's soluble starch), which was poured into boiling water and boiled for 30 min. under a reflux condenser. Gelatin solution was prepared by dissolving gelatin (Coignet Père et Cie, Paris) at 40°; the p_H of the solution was 5·89. Merck's amylase ("Diastase absolut") dissolved in saline solution and filtered was used in

these experiments. The p_H of all systems was fixed by addition of Sørensen's phosphate buffer solutions (p_H 5·89). Reducing sugar was determined by Bertrand's method and starch by Pflüger's method for glycogen.

(i) *Determination of sugar in the presence of gelatin.*

In view of the observation that the results obtained by Bertrand's method for reducing sugars are greater in aqueous than in gelatin solution, it was first necessary to determine whether the difference depended on the concentrations of sugar and of gelatin, on the time during which these were in contact, or on the temperature at which the systems were maintained.

0·3 % solutions of glucose or maltose, containing 1, 2, 3 or 4 % of gelatin, were left for 2 hours at 30°, after which the sugar content was compared with that of similar systems which had been allowed to stand for 10 min. at 15°; the results indicate that neither the duration of contact nor the temperature affects the results obtained.

The effect of varying the concentrations of sugar and of gelatin was studied in the following way (Table I). The sugar contents of 20 cc. of 0·1, 0·2, 0·3 and 0·4 % glucose or maltose in 5 % gelatin solution, and of 0·2, 0·4, 0·6 and 0·8 % maltose in 0·5 % gelatin solution were determined; in all cases the values found were lower than in aqueous solution, and the difference varied irregularly with the concentration of sugar, from 51·5 % for 0·1 % maltose

Table I. *Influence of concentration of sugar on the values found using Bertrand's method in the presence of gelatin.*

20 cc. were taken for the determination.

	% concentration of sugar												
	0·1		0·2		0·3		0·4		0·6		0·8		Conc. of gelatin %
Sugar	mg. found	% diff.	mg. found	% diff.	mg. found	% diff.	mg. found	% diff.	mg. found	% diff.	mg. found	% diff.	
Maltose	9·7	51·5	30·5	23·7	50	16·6	64	20	—	—	—	—	5
,,	—	—	39	2·5	—	—	75	6·25	83	30	142	11·2	0·5
Glucose	12·5	37·5	26·7	33·3	48	20	52	35	—	—	—	—	5

in 5 % gelatin to 20 % for 0·4 % maltose. In systems in which the concentration of sugars was maintained constant (0·3 %), but that of gelatin was varied (1·25–7·5 %), it was found that the proportion of sugars found by Bertrand's method diminished with the concentration of gelatin, the difference increasing in the case of maltose from 1·8 % in 1·25 % to 30·4 % in 7·5 % gelatin; the corresponding figures for glucose were 7·5 % and 28·3 % (Table II).

The following experiments were next performed in order to determine whether the above effect was due to combination of gelatin with sugar, with the production of a compound of lower reducing capacity, or to combination with cuprous oxide, with consequent prevention of precipitation. 15 cc. of 0·3 % maltose solution were added to 40 cc. of Bertrand's solution, and the mixture was heated to boiling. As soon as the precipitate of cuprous oxide

Table II. *Influence of concentration of gelatin on the reducing sugar content as determined by Bertrand's method.*

The sugar content was 0·3 % and 20 cc. were taken for the determination.

Conc. gelatin	mg. glucose found	% difference	mg. maltose found	% difference
0	60·0	—	57·5	—
1·25	55·5	7·5	56·5	1·8
2·5	53	11·7	55	4·3
5	48	20	50	11·1
7·5	33	28·3	40·5	30·4

had commenced to form, 5 cc. of 8 % gelatin were added to one series of flasks, and 5 cc. of water to another, after which the solutions were boiled for a further 3 min., and cuprous oxide determined as usual. Practically identical results were obtained in all cases (42 mg. where gelatin was added, and 41·8 mg. in its absence). It follows that cuprous oxide does not combine with gelatin, nor is its precipitation prevented by gelatin, under the conditions of the above experiment.

Solutions containing maltose and gelatin were next subjected to ultrafiltration at 30° through parchment membranes, using a Zsigmondy apparatus. The membranes used were not completely impermeable to gelatin; for the purpose of this experiment it was considered sufficient if the filtrate gave no precipitate on addition of alcohol, as harder filters were partially impermeable to maltose, with consequent dilution of sugar in the filtrate. The results obtained, using 0·3 % maltose in 2 % gelatin solution and taking 10 cc. of filtrate or solution for each determination, indicate that compound-formation does not take place, 34·8 mg. of maltose being found in 10 cc. of the filtrate of the gelatin solution, and 33·3 mg. in the filtrate from solutions not containing gelatin, whilst the corresponding values for unfiltered solutions are 32·0 and 38·7. The cause of the lower values obtained by Bertrand's method for reducing sugar in the presence of gelatin is not therefore apparent.

(ii) *Inhibition of action of amylase on starch.*

(*a*) *Kinetics of starch-gelatin-amylase systems.* It was at first attempted to determine the velocity of decomposition of starch by amylase, in the presence of various concentrations of gelatin, by comparing the reducing sugar content of such systems with that of systems not containing gelatin. The results obtained were, however, not reproducible, in spite of the application of every precaution, and it was found necessary in certain cases to determine not reducing sugar produced but residual starch, using Pflüger's method for glycogen.

Series of systems, containing 10 cc. of 0·5 or 1 % starch solution, 5 cc. of gelatin solution, and 1 cc. of 0·1 % amylase or water were prepared, and the starch content was determined after keeping for 2 hours at 30°. The amount of starch decomposed was 37–38 mg. in systems containing 0–4 % of

gelatin, 30·3 mg. in 5 %, 29·3 mg. in 6 %, and 24 mg. in 9 % gelatin, representing a percentage retardation of respectively 20, 22 and 36 (Table III).

Table III. *Concentration of gelatin and velocity of enzymic hydrolysis of starch.*

Conc. starch %	Conc. gelatin %	mg. starch decomposed	
		In absence of gelatin	In presence of gelatin
0·63	1	83	83
		89	88
,,	2	83	81
		89	90
,,	3	83	85
		89	89
,,	4	83	79
		89	89
,,	5	83	81
		89	87
,,	6	76	70
		84	79
,,	7	76	72
		84	78
,,	8	76	74
		84	78
,,	9	76	76
		84	79
,,	10	76	71
		84	76
0·32	1	38	38
,,	2	,,	38·6
,,	3	,,	38
,,	4	,,	37
,,	5	,,	30
,,	7	,,	29
,,	9	,,	24

(*b*) *Diffusion of starch and of amylase through gelatin gel.* Starch-gelatin gels were prepared by cooling mixtures of 15 cc. of 8 % gelatin and 10 cc. of 1 % starch to 0°, 15 cc. of water were then poured on the gels, which were left for 2, 4, 6 and 12 hrs. at 0°, after which the aqueous layer was examined for starch. This was in all cases found to be absent, indicating that starch does not diffuse through gelatin gels under the above experimental conditions. Analogous experiments in which 0·2 % amylase was taken in place of starch solution and in which the amylolytic power of the aqueous layer was determined showed that similarly amylase does not diffuse through gelatin gel.

(*c*) *Velocity of reaction in the presence of starch or amylase contained in gelatin gel.* Flasks were prepared containing 15 cc. of 3 % gelatin and 10 cc. of water or 1 % starch solution, and the solutions were left for 1, 2, 8 and 24 hrs. at 16°. 10 cc. of 1 % starch solution were then added to those flasks to which it had not previously been added, and 10 cc. of water to the remainder, and all flasks were allowed to stand for 1 hr. at 16°, after which 10 cc. of 0·1 % amylase were added to all systems. Half of the systems were stirred before addition of amylase, so as to break up the gel, which was left intact in the remainder. The starch content of all systems was determined after

standing for 1 hr. at 16°; the results, given in Table IV, show that considerable retardation (about 70 %) results from occlusion of starch within the gel, and that this retardation was considerably smaller (about 40 %) in those systems in which the gel had previously been broken up.

Table IV. *Effect of gelation of starch solution on velocity of reaction.*

	mg. reducing sugar found					
	Systems not stirred before addition of amylase			Systems stirred before addition of amylase		
Time of gelation hrs.	Starch added to gel	Starch and gelatin solutions mixed before gelation	% inhibition	Starch added to gel	Starch and gelatin solutions mixed before gelation	% inhibition
24	26·6	4·9	81·2	26·3	15·4	45·2
8	28	4·3	84·6	28·3	15·6	44·8
2	25	12	52	29·3	17·3	40·9
1	25·3	7·2	71·1	28	19·6	30·0

Similar experiments were next performed on occlusion of amylase by gelatin gels. Solutions containing 15 cc. of 3 % gelatin and 1 cc. of 0·1 % amylase or water were left for 24 hrs. at 16°, after which 10 cc. of 1 % starch solution or water were added; as in the previous experiment, the gel in half of the flasks was broken up by stirring before the addition of starch. The reducing sugar content was determined after standing for 90 min. at 16°, and amounted to 46 mg. in the control systems, to 7·6 mg. in the stirred systems, and to zero in those systems consisting of unbroken gel and amylase solution.

The velocity of reaction in gels containing both starch and amylase together was studied in the following way. A series of flasks was prepared; each contained 10 cc. of 1 % starch, 15 cc. of 0·5 or 5 % gelatin, and 1 cc. of 0·1 % amylase and was immersed in ice-water for 5 min., when the 5 % gelatin solutions set to a jelly. The flasks were then left in water at 16° for 4 hrs., after which reducing sugars were determined using Bertrand's method. 54 mg. of sugar were found in 0·5 % gelatin solutions, and 47 mg. in 5 % gelatin gels (5 experiments). This result points to the absence of retardation of reaction to any considerable extent by conversion of the medium from the liquid state to a jelly; the possibility existed, however, that this effect was only apparent, and that the reaction proceeded with great velocity in its initial phases, before gelation was complete. That this was not the case was shown by experiments in which reducing sugars were determined immediately on gelation, *i.e.*, 10 min. and 150 min. after addition of amylase, 1·8 and 40 mg. being found respectively (5 experiments).

(*d*) *Velocity of reaction in gels.* The experiments described above indicate that the velocity of reaction is chiefly influenced by the physical state (sol or gel) of one part of the system at the moment of addition of the other. The influence of mechanical breaking up of the gel was examined in the following way.

A number of systems containing 10 cc. of 1 % starch and 15 cc. of 2 % gelatin were allowed to stand for 20 hrs., during which time all systems set to a jelly. Two flasks were then shaken for 210 min., and 1 cc. of 0·1 % amylase was added, whilst amylase was immediately added to two other flasks, which were then shaken for 210 min. Amylase was also added to a third pair of flasks, which were not shaken at all and to the control systems not containing gelatin. Reducing sugar was determined in all systems, using Bertrand's method, 19·3 mg. being found in systems shaken before the addition of gelatin, 19·0 in those shaken after, 0·0 in unshaken systems, and 24 mg. in the control systems, both shaken and unshaken.

A number of similar systems were now set up, with the difference that gelation was not allowed to take place. Half the solutions were shaken for 2 hrs., and all flasks were then left for 20 hrs. at 30°, after which 1 cc. of 0·1 % amylase was added to all flasks, and reducing sugar was determined after 1 hr. at 30°. Practically the same values were found in all systems, *viz.* 46·8 and 46·0 mg. in shaken and unshaken gelatin solutions respectively, and 44 and 46 mg. in shaken and unshaken control solutions respectively.

(*e*) *Effect on amylolysis of inhibition of gelation due to addition of alcohol.* A number of systems were prepared, containing 10 cc. of 1 % starch, 5 cc. of 9 % gelatin solution or phosphate buffers at the same p_H, and 4 cc. of water or 96 % ethyl alcohol. Gelation is to a large extent inhibited by addition of alcohol. 1 cc. of 0·1 % amylase was added to all systems, and reducing sugar was determined after 4 hrs. at 17° and after 1 hr. at 30°. At 17° the control flasks without alcohol contained 59·7 mg., whilst with alcohol 42·7 mg. of sugar were found, indicating a certain inactivation of amylase by alcohol. In systems containing gelatin, those to which alcohol had not been added contained 5·3 mg., whilst in the presence of alcohol 40·2 mg. were found. At 30° gelation did not take place, and alcohol has consequently an exclusively inactivating action, 44 and 31·4 mg. of reducing sugar respectively being found in control systems without and with alcohol, and 44·9 and 32·6 mg. in the corresponding systems with gelatin.

II. Action of urease in media containing gelatin.

A number of systems were set up containing 10 cc. of 1 % urea, 5 cc. of phosphate buffer (p_H 5·9), 5 cc. of water or gelatin of the requisite concentration and 1 cc. of 0·1 % urease. The flasks were left for 1 hr. at 30°, after which ammonia was determined by Folin's aeration method. The velocity of reaction was the same in all cases, 66–68 mg. of urea being found in all systems containing 0–8 % of gelatin.

Discussion.

The velocity of diffusion is, as follows from Einstein and Smoluchowski's formula, inversely proportional to the viscosity of the medium, whence it might be expected that in certain heterogeneous systems increase in viscosity

would lead to retardation of reaction. In the case of the system amylase-0·3 % starch-gelatin, however, no retarding effect is observed below 5 % gelatin, in spite of the considerable viscosity of such systems, whilst using 0·6 % starch retardation of reaction is inconsiderable even in 10 % gelatin. This difference might be ascribed to the relatively sparser distribution of starch particles in the former case than in the latter, as a consequence of which the average distance travelled through the solution before collision with amylase particles is greater; since the time required to travel this distance is proportional to the viscosity of the medium, factors connected with diffusion have a greater influence in more dilute than in more concentrated solutions. The above results are not in agreement with the experiments described in Part VIII of this series, in which 1 % gelatin exerted a considerable inhibitory action on similar systems; the p_H of the systems studied earlier was, however, different from that maintained in the above reactions, and hence the apparent discrepancy.

In the case of the system urease-urea-gelatin, the velocity of reaction appears to be independent of the viscosity of the medium.

The experiments on jellies indicate that diffusion of colloidal particles such as starch or amylase is practically nil, *i.e.* that on the addition of a solution of one substrate to a jelly containing the other practically no reaction takes place unless the jelly is broken up. Where both enzyme and substrate are enclosed within the same jelly, the reaction velocity is little inferior to that in solutions. It is possible approximately to calculate spatial separation of the individual amylase particles; this calculation can be, of course, only relatively correct as to the order of magnitude. The systems in question contained 100 mg. of starch and 10 mg. of amylase in 16 cc. of jelly. Assuming that the given sample of amylase contains 1 % of pure enzyme, and that the mol. wt. of the latter is about 20,000, it follows that about 2×10^{16} molecules are present in the system. Taking the mol. wt. of starch to be 40,000, there should at the same time be present 10^{20} starch molecules, whence it follows that the system contains 5000 starch molecules per molecule of amylase, and that the average distance between individual amylase molecules is about $100\mu\mu$. According to Hardy [1928] the sphere of action of colloidal particles may exceed $100\mu\mu$, whence it would follow that the immobilisation of the above system need not necessarily inhibit reaction. An alternative explanation would be that amylase particles combine immediately with the entire number of starch molecules corresponding to one enzyme particle, and that subsequent gelation of the system would hence be without effect on the velocity of reaction. This explanation would, however, be incompatible with Michaelis and Menten's [1913] theory of formation of enzyme-substrate compounds, and with the values obtained for the dissociation constant of this compound. A third possible explanation would be the possibility of local thixotropic [Freundlich, 1928] liquefaction of the jelly in the vicinity of amylase particles, with consequent mobilisation of the enclosed starch.

Finally, the possibility exists that gelatin jelly is not a homogeneous mass, but that it contains lacunae filled with more liquid substance, and that amylase and starch are chiefly concentrated in these spaces. Should both amylase and starch be present in these spaces, they would be able to move in the same way as in gelatin solution, so that the reaction could proceed with considerable velocity. The next part of this series will be devoted to a closer investigation of this phenomenon.

SUMMARY.

1. The quantity of sugar found by Bertrand's method in the presence of gelatin is smaller than in its absence. The apparent diminution in sugar content increases with concentration of gelatin.

2. The velocity of reaction in the system amylase-0·6 % starch-gelatin sol is unaffected below 9 % gelatin, and appears to be little dependent on the viscosity of the medium. Where 0·3 % starch is taken, no retardation of reaction is observed below 5 % gelatin, whilst at higher concentrations of gelatin increasingly great retardation of reaction takes place.

3. The velocity of the reaction between urease and urea is the same in the presence or absence of gelatin.

4. The rate of diffusion of starch and amylase in gelatin jellies is considerably smaller than in similar sols; as a consequence the velocity of reaction in systems containing one substrate in solution and the other enclosed in a jelly is very small. It may be greatly accelerated by destruction of structure, either mechanically or by the addition of alcohol.

5. The velocity of reaction between amylase and starch in gelatin solution is little greater than in gelatin jelly.

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LXXIX. EFFECT OF ADDITION OF SALTS ON THE ISOELECTRIC POINT OF PROTEINS. I

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THE question whether the isoelectric point (I.P.) of proteins is a fixed, invariable value or not is of great importance in the study of equilibria in biological systems, as a consequence of the fact that the physical state, osmotic pressure, viscosity, coagulation, gelation, imbibition, sorption of other colloids, *etc.* of colloidal solutions depend on how far they are from the I.P., *i.e.* on the value of the charge present on the surface of the colloidal particles.

If it be accepted that, according to Hardy [1899] and Michaelis [1923], the colloidal particles are discharged at the I.P., *i.e.* that no potential difference exists between the disperse and the dispersed phases, then the normality of anions and cations should be equal ($[a^{'}] = [a^{'}]$) for ampholytes at the I.P. The I.P. would thus depend, apart from the nature of the colloid, on the presence of various substances in the system; those substances which can affect the ratio $[a^{'}]/[a^{'}]$ must alter the p_H of the I.P.

Michaelis [1923] has expressed the opinion that the p_H of the I.P. is invariable. On the other hand, it has been shown [Gerngross and Bach, 1923; Küntzel, 1929; Ostwald *et al.*, 1925; Pauli and Valko, 1929; Wilson, 1922], that the p_H of protein solutions at the I.P. may vary considerably according to the state of purity of the protein, or to the nature and concentration of salts present; the mechanism of action of mineral salts would appear to depend on inter-ionic forces and on the formation of complex salts. The explanation of the action of salts is simpler in the case of lyophobes than of lyophils; in the former case the degree of dispersion and the stability of the sol are functions solely of the charge of the particles, whilst in the latter case two factors may exist, *viz.* the affinity of certain undissociated parts of the colloid (Loeb's hydrophilic groups) for the medium, in addition to the charge of the ionised particles. The difference between lyophils and lyophobes lies therefore chiefly in the nature of the forces affecting affinity for the medium, whilst those factors which are connected with the charge are, apart from its actual origin, probably the same in both cases. The theoretical aspect of the question of the variability of the I.P. is thus the same for both types of colloid, and for this reason the same factors should in both cases affect the p_H of the I.P.

The action of salts on the charge of hydrophobe colloidal particles may

depend, in the case of anti-ions (Pauli's Gegenionen), on the concentration and the valency of the anti-ion, on the extent to which it is able to polarise the colloidal surface, on the dimensions, *i.e.* the degree of hydration of the anti-ion, on its heteropolar structure, and on changes in hydration, and hence of solubility, of the product of association; in the case of iso-ions (Pauli's Nebenionen) on the activity factor of the salt (thus the anti-ion $K^{\cdot}$ has a different activity according to the anion with which it is associated), on the formation of difficultly soluble salts and complexes, on the increase in charge of the colloidal particles, due either to the partial neutralisation of one of the charges of ampholytes, or to adsorption of iso-ions, or, finally, to changes in the lyophilic or lyophobic character of the colloid.

It is our opinion that the above postulates may, except for high concentrations of salt, be applied also to lyophilic colloids. Experiments will be described in this paper in which the action of salts on isostable types of protein, such as gelatin and serum-albumin, and on isolabile caseinogen, is studied under various conditions.

Experimental.

The experimental material taken was crystallised serum-albumin (Merck), purified caseinogen, and purified gelatin [Przyłęcki, 1930]. These substances were dissolved in such amounts that the final concentrations in the systems studied, after addition of all reagents, were 0·25, 0·5, 1·0 and 2·0 %. The p_H of the systems was adjusted by adding the requisite amount of 0·02 N, 0·1 N or N sodium hydroxide or hydrochloric acid. p_H was determined potentiometrically, using a hydrogen electrode; in certain cases it was determined before and after addition of salt, and before and after boiling. The salts used were Merck's chemically pure reagents and were added before boiling or addition of alcohol to the systems.

The action of heat was studied in the following way. Solutions of different salts were placed in a number of test-tubes of approximately equal diameter, and an equal volume of water was placed in the control test-tubes. Water was added to 5 cc. in all test-tubes, 10 cc. of serum-albumin solution were added, and the contents of all tubes were thoroughly mixed. The test-tubes were then placed in a holder, which was immersed in boiling water and left for 10 or 20 min. before removal of the systems from the water-bath. In some cases the degree of coagulation was roughly determined by filtering and comparing the intensity of the biuret coloration given by the filtrates.

The effect given by salts at laboratory temperature was studied on acid and alkaline solutions of caseinogen, prepared respectively by dissolving caseinogen in 0·03 N hydrochloric acid and 0·02 N sodium hydroxide. Different concentrations of various salts were added to 1 % solutions of this protein, and the production of opalescence, turbidity, or precipitation was noted.

The experiments performed in the presence of alcohol were carried out

in tall 200 cc. cylinders, in which 10 cc. of serum-albumin, caseinogen or gelatin solutions were placed, and 2 cc. of salt solution and water were added to all systems. Equal volumes of 90 % alcohol were then successively added to all systems, the final alcohol concentration of which was 85 %. The molar concentration of the salts present was for these systems calculated for 12 cc. of aqueous solution, without taking the volume of added alcohol into account.

A. Serum-albumin.

Effect of boiling in acid solutions.

The dependence of critical concentration (C_K) on the valency of iso- and anti-ions and on p_H was examined in the following way. Hydrochloric acid was added to 1 and 1·5 % solutions of serum-albumin (this will in future be designated as S.A.), to give a series of p_H 4·2, 3·9, 3·2, 2·6, 2·2, 1·4 and less than 1, and all systems were placed in boiling water. Coagulation was observed at p_H 4·5, and opalescence at p_H 4·2–3·9, clear solutions at p_H 3·2–1·4; at $p_H < 1$ the solutions again became turbid, and in 0·5–0·7 *M* hydrochloric acid coagulation again occurred.

Table I.

C_K for 1 % serum-albumin heated at 100°.

	p_H 4·1 concentration		p_H 3·2 concentration		p_H 2·2 concentration	
Salt	Molar	Normal	Molar	Normal	Molar	Normal
$K_3Fe(CN)_6$	<0·00005	<0·00015	0·00016	0·0005	0·0007	0·0021
Na_2SO_4	0·0024	0·0048	0·0094	0·0188	0·020	0·040
$MgSO_4$	0·0072	0·0144	0·031	0·062	0·150	0·300
$ZnSO_4$	—	—	0·045	0·090	—	—
$CuSO_4$	—	—	0·0576	0·1132	—	—
$\frac{1}{2}Fe_2(SO_4)_3$	0·017	0·034	0·060	0·120	—	—
$MgCl_2$	0·056	0·112	0·200	0·400	0·390	0·780
$CaCl_2$	0·053	0·106	0·180	0·360	—	—
$ZnCl_2$	0·110	0·220	0·270	0·540	—	—
NaCl	0·074	0·074	0·280	0·280	0·560	0·560
KCl	0·068	0·068	—	—	—	—

The addition of salts provoked coagulation at certain definite concentrations (C_K), which varied according to the p_H of the solution, being greater the further this was removed from the I.P. (*cf.* Table I).

Table I *a*.

Ratios of C_K of various salts.

Salts	p_H 4·1	p_H 3·2	p_H 2·2
$Na_2SO_4/MgSO_4$	1/ 3·0	1/ 3·3	1/ 7·5
$Na_2SO_4/ZnSO_4$	—	1/ 4·7	—
$MgSO_4/ZnSO_4$	—	1/ 1·45	—
$Na_2SO_4/CuSO_4$	—	1/ 6·1	—
$MgSO_4/MgCl_2$	1/ 8	1/ 6·5	1/ 2·6
$Na_2SO_4/NaCl$	1/16·25	1/14·9	1/14·0
$NaCl/MgCl_2$	1/ 1·5	1/ 1·4	1/ 1·4
$MgCl_2/ZnCl_2$	1/ 2·0	1/ 1·35	—
$Na_2SO_4/MgCl_2$	1/23	1/21·3	1/19·5

In Table II are given the results expressing the relation between the p_H of the solutions, the concentration of hydrochloric acid and the C_K of sodium sulphate. It will be seen that the concentrations of acid and salt are directly proportional to one another, whilst such a relation between p_H and C_K holds only over a narrow range. In strongly acid solutions (0·5–0·7 N) coagulation takes place with or without the addition of salts, but in 0·4 N HCl coagulation occurs on the addition of very small quantities of salts ($MgCl_2$, NaCl, or Na_2SO_4). The curves connecting C_K of salt with p_H of solution exhibit a maximum at p_H 1·4–2·2.

Table II.

C_K of Na_2SO_4 at various p_H and concentrations of hydrochloric acid.

Conc. of HCl M	0·009	0·0167	0·0227	0·0286	0·050	0·500
p_H	3·99	3·22	2·60	2·20	1·40	1
C_K Na_2SO_4 (M)	0·0046	0·0114	0·017	0·0205	0·040	0·003
$[HCl]/[Na_2SO_4]$	2·0	1·5	1·3	1·4	1·25	170·0

The salts studied can be classified into a number of groups, according to the valency of the anion; thus potassium ferricyanide in concentrations of less than 0·001 M produces coagulation at 16°, but is slightly less active at 100°, whilst the bivalent sulphate ion has a much weaker action, and using chlorides (NaCl, $MgCl_2$, $CaCl_2$) C_K is so high that the dehydration factor has to be taken into account in addition to the ionic effect. The following series of C_K is obtained for ter-, bi-, and univalent anions:

p_H 4·1	$Fe(CN)_6'''$: SO_4'' : Cl'	= 1 : 32 : 520
p_H 3·2		1 : 31·3 : 470
p_H 2·2		1 : 20 : 350
p_H 4·1	SO_4'' : Cl'	= 1 : 16·3
p_H 3·2		1 : 14·9
p_H 2·2		1 : 14·0

Apart from the above relations, which are in agreement with Hardy's and Schulze's anti-ion valency rule, a very interesting relation between the nature of the iso-ion and the C_K of the anti-ion is apparent. If we compare the value of C_K for sodium, magnesium, and ferric sulphates we see that at p_H 4·1, for example, it is lowest for sodium sulphate (0·0024 M), three times as great for magnesium sulphate (0·0072 M), and over seven times as great for ferric sulphate (0·034 M). For solutions the p_H of which is further removed from the I.P. these differences increase, until at p_H 2·2 we have the ratio $Na_2SO_4 : MgSO_4 = 1 : 7·5$. The same phenomenon holds, although to a less marked extent, for salts possessing univalent anions; thus at the same p_H C_K is 1·4 times as great for magnesium as for sodium chloride. The action of bivalent cations was further studied using copper and zinc sulphates, and zinc and cadmium chlorides. The results obtained with zinc and copper salts are of the same order as those obtained using alkaline earth salts, whilst cadmium chloride has a considerably higher C_K than have other salts possessing a bivalent cation.

Only very small changes in p_H are noticed as a result of coagulation of

protein. Thus, in one case, the p_H of a control system containing serum albumin, acid and water was 3·02 both before and after boiling, and the p_H of the same system with sodium sulphate was 3·26 before boiling, and 3·30 after.

Table III.

Influence of dilution of albumin on the C_K of sodium sulphate.

% albumin	2	1	0·5	0·25
C_K	0·0094 *M*	0·0078 *M*	0·0062 *M*	0·0054 *M*

If coagulation is brought about by the presence of definite concentrations of salt, dilution of the whole system before boiling should inhibit coagulation. This was found to be the case; the addition of an equal volume of water at the same p_H as that of a system in which coagulation occurred after heating for a few minutes at 100° prevented coagulation. In connection with these experiments, it was noticed that the C_K of salts diminishes with the dilution of albumin (Table III).

Dependence of C_K for sodium sulphate on the peptising acid.

The results given in Table IV show that the C_K of sodium sulphate is considerably higher for hydrochloric acid than for sulphuric acid solutions of albumin (0·020 and 0·012 *M* respectively). This difference is due partly to the coagulating effect of the sulphate ions originating from the sulphuric acid originally present in the second case: it is, however, of interest that the total sulphate ion concentration is greater at coagulation for such systems than for hydrochloric acid solutions, and the difference between the action of the SO_4'' from sulphuric acid and from sodium sulphate increases with greater original concentration of sulphuric acid (Table IV).

Table IV.

C_K of sodium sulphate in acidified systems, containing 50 cc. of 1 % serum-albumin and 30 cc. of 0·1 *N* acid.

	M conc. of Na_2SO_4 in the presence of	
Solutions are	H_2SO_4	HCl
Clear	0 –0·005	0 –0·0142
Opalescent	0·005–0·012	0·0142–0·023
Coagulated	Over 0·012	Over 0·023

Simultaneous action of different anti- and iso-ions.

The addition of sodium sulphate up to a definite concentration to solutions of serum-albumin in the presence of sub-critical concentrations of potassium ferricyanide leads to coagulation, even in unheated solutions. This effect is due to summation of the action of different multivalent anti-ions, and the C_K of the second salt in the presence of the first is greater, the greater the dilution of the first below its C_K (Table V).

Table V.

Coagulative effect of Na_2SO_4 in the presence of sub-critical concentrations of ferricyanide at p_H 2.

M concentration of Na_2SO_4	No $K_3Fe(CN)_6$	0·0004 M $K_3Fe(CN)_6$	0·0006 M $K_3Fe(CN)_6$
0	Clear	Clear	Slight opalescence
0·017	,,	Opalescent	Flocculation
0·025	,,	Intense opalescence	,,
0·050	,,	Flocculation	,,

The addition of magnesium or aluminium chloride to systems containing sodium sulphate, keeping the p_H and volume constant, has a contrary effect, raising the C_K of the latter salt. The magnitude of this effect depends on the p_H of the systems, on the concentration of salt, and on the valency of the iso-ion (Table VI). Thus, adding various quantities of magnesium chloride

Table VI.

Peptising effect of various iso-ions in systems containing 0·030 M Na_2SO_4 at p_H 2·2.

	M conc. of added salt			
Solutions are	NaCl	$MgCl_2$	$FeCl_3$	$AlCl_3$
Flocculated	0·057–0·57	0 –0·042	0 –0·020	0 –0·010
Opalescent	—	0·042–0·083	0·020–0·035	0·010–0·021
Clear	—	Over 0·083	Over 0·035	Over 0·021

to a 1 % albumin solution at p_H 2·65, the maximum elevation of C_K for sodium sulphate occurs in 0·1 M $MgCl_2$, whilst at p_H 3·6 the maximum C_K is in 0·05 M $MgCl_2$. In general, the peptising action of magnesium chloride diminishes as the p_H of the solution differs more and more from the I.P. The action of aluminium chloride is considerably greater than is that of magnesium chloride, since the latter salt under optimal conditions increases the C_K of sodium sulphate 2·5 times, and the former 6·7 times (Table VII). At the same

Table VII.

Peptising action of $MgCl_2$ and $AlCl_3$.

	C_K of Na_2SO_4 at			C_K Na_2SO_4 at
M conc. of $MgCl_2$	p_H 3·6 (M)	p_H 2·65 (M)	M conc. of $AlCl_3$	p_H 3·0 (M)
0	0·0064	0·0161	0	0·010
0·025	0·014	—	0·007	0·0175
0·050	0·020	0·031	0·010	0·022
0·100	0·0177	0·037	0·015	0·026
0·150	—	0·030	0·030	0·055
0·200	0·0155	—	0·055	0·067
0·250	—	0·024	—	—

time we see that the C_K of magnesium chloride is greater than that of aluminium chloride (0·08 and 0·05 M respectively, at p_H 3·2). Similar results for the peptising effect of these two salts were obtained for systems coagulated in the cold by the addition of ferricyanide (Table VIII).

Table VIII.

Peptising action of iso-ions in 2 % serum-albumin systems containing ferricyanide at p_H 2·0 and 20°.

M conc. of ferricyanide	Effect on solutions of addition of					
	H_2O	0·03 M HCl	0·3 M HCl	0·6 M NaCl	0·09 M $MgCl_2$	0·05 M $AlCl_3$
0·0004	Clear	Clear	Opalescence	Opalescence	Clear	Clear
0·0008	Opalescence	Opalescence	Turbidity	Turbidity	,,	,,
0·0012	Flocculation	Flocculation	Flocculation	Flocculation	Opalescence	Opalescence
0·0016	,,	,,	,,	,,	,,	,,
0·0020	,,	,,	,,	,,	Turbidity, then flocculation	,,
0·0024	,,	,,	,,	,,	Flocculation	Flocculation
0·0030	,,	,,	,,	,,	,,	,,

Effect of boiling in alkaline solution.

The action of the salts NaCl, $MgCl_2$, $CaCl_2$, $MgSO_4$, $AlCl_3$ and $FeCl_3$ on serum-albumin solutions at p_H 9·2 was examined. Aluminium and ferric chlorides in higher concentrations were to a small extent precipitated by alkaline solutions. Normal sodium chloride did not coagulate alkaline albumin even on boiling, whilst coagulation took place in 0·026–0·027 M calcium or magnesium chlorides without heating. The C_K of magnesium sulphate is 0·033 M, *i.e.* 27 % greater than for calcium chloride. The C_K of aluminium and ferric chlorides could not be determined with any accuracy, and we do not attach any special significance to our figures (Table IX).

Table IX.

C_K of various salts for 1 % serum-albumin solutions at p_H 9·2.

NaCl	$CaCl_2$	$MgCl_2$	$MgSO_4$	$AlCl_3$	$FeCl_3$
0·640 M (opalescence)	0·026 M	0·027 M	0·033 M	0·0045 M	0·006 M

The C_K of magnesium chloride was determined alone and in the presence of sodium chloride or sulphate and potassium ferricyanide. All systems were at p_H 9·2, and contained 1 % serum-albumin, and 0·03 M $MgCl_2$, and were, in the absence of other salts, in a state of flocculation. The addition of 0·014 M Na_2SO_4 or of 0·0016 M $K_3Fe(CN)_6$ prevented flocculation; the systems exhibited, however, intense opalescence. As the concentration of salts is augmented, the solutions become increasingly clear, so that in the presence of 0·03 M Na_2SO_4 or 0·003 M $K_3Fe(CN)_6$ they are practically quite clear (Table X).

Table X.

C_K for $MgCl_2$ at p_H 8·5 (M).

	$MgCl_2$ alone	In presence of NaCl			In presence of Na_2SO_4		
		0·15	0·30	0·55	0·0135	0·020	0·025
C_K $MgCl_2$	0·025	0·0263	0·0257	0·022	0·0335	0·035	0·032

Precipitation by alcohol.

The relation between the C_K of sodium sulphate, the p_H of the system, and the concentration of hydrochloric acid was determined in the presence of 85 % ethyl alcohol. The results, given in Table XI, show that a proportionality exists between the concentration of acid and the C_K of sodium sulphate.

Table XI.

Comparison of C_K of Na_2SO_4 for boiled systems and for 85 % alcoholic solutions.

p_H	C_K for systems heated at 100° (M)	C_K for 85 % alcohol systems (M)	$\frac{C_K\ 100°}{C_K\ \text{alcohol}}$	$[Na_2SO_4]/[HCl]$ 100°	$[Na_2SO_4]/[HCl]$ Alcohol
3·2	0·0096	0·0016	6·0 : 1	1·6	9·6
2·6	0·0145	0·0027	6·1 : 1	1·5	10·0
2·0	0·0223	0·0042	5·3 : 1	1·75	11·0
1·6	0·031	0·0057	5·5 : 1	1·6	10·0
1·4	0·042	0·009	4·7 : 1	1·5	8·4

Comparison between the C_K of sodium sulphate in systems coagulated by heat and by alcohol.

C_K was determined for sodium sulphate in acid serum-albumin solutions, in the presence of 85 % alcohol, and heated at 100°. The value of C_K is at p_H 3·2 six times greater in the latter case than in the former, but the difference diminishes as more acid solutions are taken, so that at p_H 1·6 the ratio is 5:1. Acidification of the solutions to a certain extent augments C_K for both systems.

B. Caseinogen.

C_K was determined for sodium sulphate and potassium ferricyanide in caseinogen solutions at p_H 2, in unheated and heated systems and in the presence of 85 % alcohol. The results, given in Table XII, show that C_K is 0·0076 M in unheated systems, 0·0064 M at 100°, and 0·005 M in the presence of alcohol. The ratio of the C_K values in heated and alcoholic systems is 1·27, in unheated and heated systems 1·19, and in unheated and alcoholic solutions 1·52; these ratios differ considerably from those obtained for serum-albumin.

A second series of experiments on the relation between p_H and C_K showed that the C_K of sodium sulphate increases with the charge on the colloidal particles.

Table XII.

C_K of sulphate ions for caseinogen solutions acidified with hydrochloric acid to p_H 2 (M).

At 15°	Heated 10 min. at 100°	In 85 % alcohol
0·0076	0·0064	0·005

Determination of the C_K of sulphate ion added as a mixture of hydrochloric and sulphuric acids and of hydrochloric acid and sodium sulphate showed that in the former case it has a value of over 0·08 M, whilst in the latter it is 0·0067 M.

C. Gelatin.

Systems heated at 100°.

The addition of salts giving rise to univalent or bivalent ions (0·001–0·2 *M*) to gelatin solutions at p_H 1–12 does not produce coagulation, either in heated or unheated systems. Potassium ferricyanide coagulates in 0·0005–0·001 *M* solutions, according to the p_H, whilst ferrocyanide is without action.

Action of salts in the presence of alcohol.

The results for systems containing 80 % alcohol (Table XIII) are in every way similar to those obtained for serum-albumin. The C_K of sodium sulphate in systems containing 0·5 % gelatin and 50–90 % alcohol falls at first rapidly with increasing concentration of alcohol, from 0·08 *M* in 50 % to 0·0008 *M* in 85 % alcohol, above which concentration no further change is observed (Table XIV). The value of C_K rises with increasing acidity from 0·00045 *M* in 0·005 *M* HCl to 0·009 *M* in 0·06 *M* HCl (Table XV).

Table XIII.

C_K of salts in gelatin solutions containing 80 % alcohol (dilution by alcohol taken into consideration) (*M*).

p_H 7·30			p_H 1·75			
KCl	$MgCl_2$	$AlCl_3$	NaCl	Na_2SO_4	$MgSO_4$	$K_3Fe(CN)_6$
0·017	0·0025	0·00025	Over 0·15	0·003	0·0014	0·00015

Table XIV.

Relation between C_K and concentration of alcohol.

C_K of Na_2SO_4 (*M*)	% alcohol	C_K of $MgSO_4$ (*M*)	% alcohol
0·0008	85	0·011	71
0·0016	80	0·014	69
0·0032	71	0·016	66
0·0047	66	0·020	62
0·0064	62		
0·008	59		
0·032	55		
0·040	54		
0·080	52		

Table XV.

C_K of Na_2SO_4 at various p_H.

p_H	C_K of Na_2SO_4 (*M*)
3·8	0·00045
3·0	0·0012
2·5	0·0021
2·2	0·0027
1·5	0·009

Action of iso-ions.

The action of iso-ions is illustrated by experiments in which the C_K values of sodium and magnesium sulphates in the presence of alcohol were compared.

In 66 % alcohol the value of C_K was 0·0047 M for sodium, and 0·016 M for magnesium sulphate, whilst in 71 % alcohol the corresponding values were 0·0032 M and 0·011 M (Table XIV).

Table XVI.

Peptising action of $MgCl_2$ in various concentrations of alcohol.

Salts present	Concentration of alcohol necessary for flocculation %
0·0047 M Na_2SO_4	66·5
0·008 M Na_2SO_4	60·0
0·008 M Na_2SO_4 + 0·1 M $MgCl_2$	73·0
0·016 M Na_2SO_4 + 0·1 M $MgCl_2$	70·5
0·024 M Na_2SO_4 + 0·1 M $MgCl_2$	69·5
0·032 M Na_2SO_4 + 0·1 M $MgCl_2$	66·5

When magnesium chloride is added in addition to sulphate, the C_K of the latter rises, from 0·011 M in 71 % alcohol to 0·016 M in the presence of 0·05 M $MgCl_2$ (Table XVI); this effect may be ascribed to the more powerful peptising effect of the magnesium iso-ion in the presence of the feebly coagulating chloride ion. The peptising effect of magnesium ions is not evident in systems containing over 80 % of alcohol; thus in 83 % alcohol 0·002–0·003 M $MgSO_4$ coagulates gelatin, and the addition of magnesium chloride (0·01–0·05 M) does not affect the results obtained.

Similar results were obtained for systems containing sodium sulphate, magnesium chloride and alcohol. The C_K of sodium sulphate is greatly increased by the presence of 0·1 M $MgCl_2$ in low concentrations of alcohol, but less so in higher concentrations; thus in 66 % alcohol C_K is raised from 0·0044 to 0·032 M, whilst in 71 % alcohol the corresponding values are 0·0032 and 0·007 M, and in 85 % alcohol peptisation due to magnesium ions is entirely absent, only the feebly coagulating effect of chloride ions being superimposed on that of sulphate ions.

Discussion.

The stability of protein solutions depends on two factors—the affinity to water of the undissociated part of the protein molecule, and the charge of the colloidal particles. Proteins have been classified by Wo. Ostwald [1920] into isostable and isolabile; at the I.P. the affinity of the first for water exceeds their critical hydration value [Kruyt and de Jong, 1928], so that stable solutions of such proteins may be obtained without the addition of peptisers. Isostable proteins may be further classified into thermo-isostable and thermo-isolabile, according to the behaviour of their solutions on heating; should the affinity for water fall below the critical value at higher temperatures thermo-isolabile proteins coagulate. The affinity to water of isolabile proteins is even at the ordinary temperature less than the critical, so that the stability of sols of such proteins is a function of the charge on the particles. No sharp division exists between isostable and isolabile proteins; the thermo-isolabile group

represents a transition form between the two groups and certain of the thermo-isolabile proteins may at the I.P. be considered to be in a state of slow coagulation.

Isostable proteins may be obtained in the isolabile state, either by heating at above the critical temperature, in the case of thermo-isolabile proteins, or by the addition of such organic solvents as alcohol, acetone, ether, chloroform, *etc.* In the case of systems rendered isolabile by the addition of alcohol, two interesting observations, evidencing the relationship between the critical concentration of alcohol and the affinity of the given protein for water, are to be noted. These are the behaviour of purified protein at the I.P., and the difference between the action of electrolytes on protein solutions at p_H values other than the I.P. of the given protein and at the same p_H in the presence of alcohol. At the I.P. the C_K of alcohol increases with the isostability of the given protein, so that, *e.g.*, gelatin at the I.P. is coagulated by about 48 % alcohol, whilst serum-albumin is coagulated by 36 % alcohol.

At p_H values other than that of the I.P. the properties of various proteins with respect to the behaviour of their aqueous solutions on the addition of salts approach more nearly to one another as the concentration of alcohol in the system rises. Thus at p_H 2·5 aqueous gelatin is precipitated by 0·6M, serum-albumin by 0·5M, and caseinogen by 0·0076M Na_2SO_4. In heated solutions, the corresponding values of C_K are respectively 0·55, 0·031 and 0·0064M; here the C_K of sodium sulphate for serum-albumin is very close to that for caseinogen. Finally, in 85 % alcohol, the values of C_K are respectively 0·006, 0·0057 and 0·005M, showing that in these conditions the properties of these apparently so different proteins have approached closely to one another.

As the concentration of alcohol rises, the charge on the colloidal particles has to be increased in order to maintain solution. Coagulation may be effected at any p_H using the same concentration of alcohol as at the I.P., by adding a critical concentration of electrolyte. As the concentration of alcohol rises, dehydration increases, with consequent diminution in C_K of the electrolyte, which varies from 0·08M in 50 % to 0·0018M in 90 % alcohol. This phenomenon might be interpreted variously. Thus it might be supposed that even in high concentrations of alcohol gelatin still possesses a certain affinity for water, and that hydration remains as one of the stabilising factors. The stabilising effect of hydration may, however, be in itself insufficient to prevent coagulation without the additional action of a charge on the particles, the neutralisation of which would therefore lead to coagulation. In connection with this theory it would be necessary to assume that the particles are not completely discharged at the C_K, and that, as a result of a certain residual affinity for water, stabilisation is the resultant of the summation of two factors. For this reason the greater the degree of dehydration, the more the C_K of sodium sulphate approaches that for systems the stabilisation of which depends exclusively on the charge. Various other explanations might be put

forward; in the present state of knowledge of the subject, however, these would be somewhat speculative. These explanations would include possible variations in the thickness of the electrical double layer, decrease in the charge, changes in the dielectric constant and in the activity factor of the salts, and augmented association.

The observations recorded in this paper indicate that the dispersion of proteins in colloidal solution is due to two factors, which have to be reduced to a certain minimum for coagulation to supervene. This view is based on the following grounds. For isolabile proteins their charge is the decisive factor for the stabilisation of the sol. Since the charge is a consequence of the structure of both isolabile and isostable proteins, there is no reason for supposing that the latter class are not charged in solution. The C_K of various salts for isolabile proteins are very similar in ordinary and in dehydrated solutions (boiled, or in the presence of alcohol); in such solutions the C_K for iso-labile and -stable proteins are very close to one another, suggesting that such factors as alcohol or heat convert isostable proteins into a form similar to the isolabile. This process depends either on the action of the factors applied on groups on which depends the affinity of the given protein for water, or on changes in the concentration or physical state of water (*e.g.* increased mobility at 100°). At the same time the nature of the factors to which the charge on the particles is due does not undergo change, with the exception of variation in the thickness of the double layer and in its composition, and for this reason the dehydrated form of isostable proteins is well adapted to the study of variations in the charge.

This depends on the ratio of anions to cations; when these are present in equivalent concentrations, the charge is zero. If it be accepted that the charge is connected with the f_a of the dissociated salt and with the degree of dissociation of the colloid, as well as, within certain p_H limits, with zwitterion dissociation, the action of ions will depend on the degree of association and the f_a of the salts produced. These factors are the same as those taking part in homogeneous systems; in protein solutions the charge on the particles is further affected by surface forces, and, above all, by the thickness of the double layer.

The observed facts might be given a different interpretation from that advanced above; it might be considered that ions, in particular anti-ions, form salts with protein ions, and that the solubility of certain of the salts thus formed is less than that of the free protein. The coagulative action of salts would then depend not on their action on the charge on the particles but on the formation of difficultly soluble salts. This explanation is, however, improbable, in view of the following facts. In a relatively dehydrated medium containing alcohol, the C_K of salts for different proteins have very similar values, in spite of the fact that the solubilities of these proteins and probably therefore of their salts are very different. Further counter-arguments are that the action of ions is connected with their valency, that iso-ions act in the

opposite way to anti-ions, that the closer the system is to the I.P., the smaller is C_K, and that the C_K of sulphate ions is at the same p_H 4–6 times greater when they are added as sulphuric acid than when added as sodium sulphate.

All these observations are in agreement with the hypothesis that the action of salts is due to the action of their ions on the charge of the protein particles. Thus the action of the anti-ions conforms to the rule of Hardy and Schulze whereby the action of discharging anti-ions is a function of their valency. In our case, the ratio of the action of uni-, bi-, and tervalent anions is as 1:30:500; these ratios are similar to those found [Pauli and Valkó, 1929] for lyophobe gold sol (1:30:250). The connection between the action of anti-ions and the charge on the colloidal particles is shown by the observation that C_K rises as the p_H of the solution diminishes below the I.P., *i.e.* as the charge increases. The difference between the actions of sulphuric acid and sodium sulphate is due to their different actions on the double layer. Thus sulphuric acid up to a certain concentration augments the positive charge of protein, owing to the action of its cations, whilst the action of sulphate is due almost exclusively to its anions. The action of iso-ions is complex, and depends on the degree of dehydration of the colloid; where this is incomplete iso-ions inhibit the coagulative action of anti-ions, to an extent depending on their valency. The action of uni-univalent salts appears to be due chiefly to displacement by univalent of bivalent iso-ions from acid albuminates, whilst where multivalent iso-ions are added to systems containing multivalent anti-ions the increase in the C_K of the latter is due partly to reactions of double decomposition, resulting in products of a lower f_a; thus the addition of magnesium chloride to sodium sulphate yields a certain amount of magnesium sulphate, the f_a of which is considerably lower than that of sodium sulphate. A further effect is that of attraction by iso-ions of anti-ions from the hydrosphere of the protein particles to the bulk of the solution, with consequent augmentation of the free charge of the protein complexes.

From the point of view of the zwitterion theory, the antagonistic effect of iso-ions in the coagulation of protein by addition of anti-ions may depend on the formation of salts of lower f_a between the carboxyl residues and the iso-ions, thereby augmenting the free positive charge due to ionisation of the amino-groups; this would apply to systems at the p_H of the I.P., or slightly below this p_H. This view is supported by Loeb's [1924] experiments, which showed that the free charge of denatured proteins is increased by the addition of salts yielding multivalent iso-ions and univalent anti-ions. According to Dorfmann [1930] multivalent iso-ions may further affect the degree of hydration of colloids, in this way inhibiting coagulation.

In systems dehydrated by the addition of alcohol the action of multivalent iso-ions diminishes with increasing alcohol content; no obvious explanation of this phenomenon is apparent.

It appears from the work of Loeb [1924, 1925] and of Kruyt *et al.* [1928, 1929], as well as from the results described in the present paper, that the chief

factor in the coagulative action of electrolytes on proteins is the reduction of the free charge on the colloidal particles below a certain critical minimum. A certain difference exists between proteins discharged at the isoelectric point and at other p_H, due to the fact that on the alkaline side of the I.P. the particles are discharged by and consequently associated with cations, and on the acid side by anions; the conception of the isoelectric state would apply only to purified proteins of zero free charge, whilst proteins discharged by electrolytes at other p_H values than that of the I.P. should rather be termed electrically neutral at the given p_H.

Summary

1. Serum-albumin solutions coagulate on heating at 100° at p_H above 4·7 or below 1.

2. Various mineral salts produce coagulation at a certain critical concentration (C_K) for each p_H. The C_K is at a maximum at p_H about 2, and varies in accordance with the Hardy-Schulze rule. The ratios of C_K for ter-, bi-, and univalent anti-ions are as 1:30:500.

3. The value of the C_K of anti-ions also depends on the valency of the accompanying iso-ion.

4. The value of C_K of salts depends on the nature of the peptising acid; it is less in solutions acidified with acids giving rise to bivalent than to univalent anti-ions.

5. The coagulative action of two or more anti-ions at concentrations below the critical is additive.

6. Salts possessing a univalent anti-ion and a multivalent iso-ion exert in certain concentrations a peptising action.

7. Analogous results to the above are obtained at p_H values higher than those of the isoelectric point, at which cations are anti-ions.

8. Coagulation can be effected by the addition of salts to systems containing 85 % alcohol at any p_H. The Hardy-Schulze rule is followed in such systems, and the maximum values of C_K are at p_H 2.

9. The C_K of sodium sulphate for caseinogen hydrochloride at p_H 2 has practically the same value at 20°, 100°, and in 85 % alcohol systems (respectively 0·0076 M, 0·0064 M and 0·005 M).

10. The C_K of the sulphate ion added as sulphuric acid is considerably higher, *viz.* over 0·1 M.

11. Gelatin in 80 % alcohol can be flocculated at any p_H by critical concentrations of anti-ions, the values of C_K varying according to the Hardy-Schulze rule. The value of C_K varies inversely with the concentration of alcohol.

12. The C_K of sodium sulphate increases with the free charge on the gelatin particles up to a certain maximum, after which it again falls, as for serum-albumin.

13. The C_K of anti-ions increases with the valency of the iso-ions. The critical concentration of alcohol necessary to flocculate gelatin at a given concentration of sodium sulphate is higher in the presence of magnesium chloride than in its absence, and conversely, at a given concentration of alcohol, the C_K of sodium sulphate is considerably higher in the presence than in the absence of multivalent iso-ions.

14. The peptising action of multivalent iso-ions is not apparent in high concentrations (85 %) of alcohol, in which salts possessing a multivalent iso-ion and univalent anti-ions exert a coagulative action.

15. Isostable proteins can by heating at 100° or addition of alcohol be converted into proteins of the isolabile type. The C_K of sodium sulphate is at the same p_H practically the same for serum-albumin, gelatin, and caseinogen in presence of hydrochloric acid in 85 % alcohol.

16. Salts possessing multivalent anti-ions are able to discharge protein particles at any p_H, C_K being different at each p_H for each salt. The coagulum so obtained differs from that obtained at the isoelectric point.

17. The above observations may be readily explained if it be assumed that the state of dispersion of proteins is a function of their affinity for water and of the free charge on the particles; the reduction of the latter below a certain critical minimum results in conversion of protein sols into gels. Flocculation cannot be explained as being due to the formation of variously soluble salts.

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LXXX. THE PHOSPHATASE ACTIVITY OF TRANSPLANTS OF THE EPITHELIUM OF THE URINARY BLADDER TO THE ABDOMINAL WALL PRODUCING HETEROTOPIC OSSIFICATION.

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The presence of an enzyme capable of hydrolysing certain phosphoric esters has been demonstrated in bone and ossifying cartilage by Robison [1923], who with his collaborators has presented evidence that it is an active agent in ossification.

In general, this evidence has been adduced from three lines of observations: (*a*) survey of the normal distribution of the enzyme; (*b*) study of calcification in bone and cartilage in experimental rickets; (*c*) tissue culture *in vitro* of cartilage and bone. Some of the more important observations may be briefly cited. Normally phosphatase was found to occur in the ossified portions of young human embryos and infants, while non-ossifying cartilage was in all cases devoid of it [Martland and Robison, 1924]. When severely rachitic bones were immersed in solutions of calcium hexosemonophosphate or glycerophosphate, deposition of calcium phosphate took place in the hypertrophic, but not in the small-celled, or articular cartilage [Robison and Soames, 1924, 1930]. The addition of very small amounts of a phosphoric ester to solutions of calcium salts and inorganic phosphate in concentrations otherwise inadequate for deposition of calcium phosphate in rachitic bone led readily to calcification [Robison and Soames, 1930; Robison, Macleod and Rosenheim, 1930]: desiccation for 24 hours or the addition of such protoplasmic poisons as chloroform, acetone or potassium cyanide inhibited calcification in inorganic solutions but did not destroy the enzyme, and calcification resulted in the presence of phosphoric ester [Robison, Macleod and Rosenheim, 1930]. In tissue culture of the embryonic femur and mandible of the chick, phosphatase could be demonstrated in large amounts when hypertrophic cartilage and bone were present; in the absence of these and in certain areas which do not normally ossify in these structures phosphatase was not present in detectable amounts [Fell and Robison, 1929, 1930].

[1] The surgical work herein reported was carried out in the Department of Physiology, University College, London.

An osteogenic effect has been demonstrated in the epithelium of the urinary bladder, ureter and kidney pelvis of the dog [Huggins, 1930, 1, 2; 1931]. When, for example, a portion of the bladder epithelium is transplanted to the connective tissue of the abdominal wall, ossification occurs regularly after 18 to 20 days in the connective tissue, provided the epithelium survives. The bone so formed has a constant relationship to the epithelium; the epithelium never ossifies. The bone gives the typical crystallographic appearance of true bone when examined spectroscopically by means of the X-rays [Roseberry, Hastings, and Morse, 1931].

The object of the present experiment was (*a*) to ascertain if phosphatase occurs normally in the urinary bladder epithelium or fibrous tissue of the abdominal wall and whether it is synthesised in the bone formed by transplantation of the former to the latter, and (*b*) to study the progress of its synthesis in relation to the ossification.

Methods.

Surgical. Under aseptic precautions and ether anaesthesia the abdominal cavity was opened through a small lower mid-rectus incision, and the urinary bladder delivered through the wound. A preliminary incision was made in the dome of the bladder down to the mucosa, and a portion of the epithelium, approximately 2·5 × 2·5 cm., stripped from the muscular wall by blunt dissection and cut away. The wound in the dome of the bladder was closed with a fine silk suture, in two layers. The excised epithelium was transplanted to the top of the sheath of the rectus abdominis muscle, sewed in at four corners with a silk interrupted suture. The abdominal wall was closed, without drainage, with three silk sutures.

At operation small portions of the whole thickness of the bladder wall, the epithelium alone, the muscular wall of the bladder alone, and the connective tissue of the rectus sheath were excised for chemical examination as control specimens. The dogs were killed at intervals of 5, 9, 13, 16, 18 and 26 days after operation. At necropsy, many samples of tissue in the region of the transplant were removed and divided into nearly equal portions, the one half for histological examination and the other for phosphatase determinations.

Chemical. The method for determination of phosphatase activity was similar to that employed by Fell and Robison [1930]. The tissue was transferred to a cover-slip and dried for 18 hours over sulphuric acid in an evacuated desiccator. It was then weighed on a micro-balance after attaining constant weight in air and transferred to a tube containing 2 cc. of water and a small drop of chloroform. The cover-slip was then reweighed and the weight (W) of tissue thus obtained. The tissues were kept in the chloroform water at room temperature for 3 days; 1 cc. of 0·2M solution of sodium glycerophosphate (p_H 8·7) was added together with a small drop of chloroform. The further procedure and calculation of the index (A/W) of phosphatase activity

has been described by Fell and Robison [1929]. The ratio A/W expresses the amount of hydrolysis as mg. P effected by 1 mg. of tissue (dry weight) at a temperature of 37° and p_H 8·6.

Results.

In all cases the transplants survived as proved by histological examination and presented the characteristic histological appearance already described [Huggins, 1931]. In the 16-day specimen "osteoid" tissue or fibroblasts with large, swollen, deeply staining cytoplasm surrounded by eosinophilic oedematous tissue was found near the transplanted epithelium: no bone was found at this time or before. In the 18- and 26-day specimens, a rather large amount of bone was found adjacent to the transplanted epithelium. This had the characteristic features of membrane bone.

In the preliminary control tissues (Table I) phosphatase activity could not be demonstrated in the fibrous tissue of the rectus sheath or the muscular portion of the bladder wall. A definite but small value (about 0·03) for A/W was obtained for the bladder epithelium. Variations between A/W values of 0·01 and 0·03 in the whole bladder wall can probably be explained by variations in the amount of epithelium included, since the muscle does not contain the enzyme.

Table I. *Phosphatase activity of various tissues removed at operation as controls.*

Tissue	W mg.	A mg. P	A/W
Whole thickness of bladder wall	3·39	0·11	0·03
,,	19·1	0·05	<0·01
,,	14·2	0·10	<0·01
Bladder epithelium alone	5·04	0·14	0·03
,,	4·5	0·10	0·02
,,	3·7	0·09	0·03
,,	3·0	0·05	0·02
,,	3·6	0·12	0·03
,,	3·7	0·55	0·01
,,	13·0	0·06	<0·01
Bladder musculature alone	6·6	0·02	<0·01
,,	9·3	0	0
Connective tissue rectus sheath	10·6	0·01	<0·01
,,	3·5	0	0
,,	5·9	0	0
,,	8·9	0	0
,,	5·7	0	0

Of the tissues of the transplant containing bladder epithelium histologically (Table II *b*) no activity could be demonstrated at 5 or 9 days. In similar specimens at 12, 13 and 16 days small amounts of phosphatase were demonstrated and the A/W value was of the order of 0·01 except in one instance where it was 0·05.

Specimens of connective tissue adjacent to but not including bladder epithelium (Table II *a*) from 12 to 26 days contained even smaller amounts of the enzyme, ranging from 0 (14 times) to a maximum value of 0·05 (once).

Table II.

Phosphatase activity of connective tissue adjacent to bladder epithelium transplant.

Time after operation	*W* mg.	*A* mg. P	*A/W*
(*a*) *No bladder mucosa or bone present.*			
12	5·0	0·25	0·05
12	7·0	0·07	0·01
13	19·7	0·25	0·01
13	22·2	0·51	0·02
13	13·2	0·06	<0·01
16	36·0	0·02	<0·01
16	12·0	0·20	0·02
16	42·0	0·005	<0·01
16	14·0	0·05	<0·01
16	9·0	0·13	0·01
18	5·07	6·192	0·04
18	6·0	0·187	0·03
18	2·7	0	0
18	12·0	0	0
18	2·0	0	0
18	4·2	0	0
26	3·6	0	0
26	4·4	0	0
26	2·2	0	0
26	1·4	0·042	0·03
26	7·0	0·033	<0·01
26	5·2	0·033	<0·01
26	4·0	0·055	0·01

Phosphatase activity of bladder epithelium transplanted to connective tissue.

Time after operation	*W* mg.	*A* mg. P	*A/W*
(*b*) *Bladder mucosa but no bone present.*			
5	1·8	0	0
5	3·0	0	0
9	3·8	0	0
9	3·3	0	0
9	5·9	0	0
12	10·0	0·113	0·01
12	11·0	0·09	<0·01
12	23·0	0·22	0·01
12	4·5	0·055	0·01
13	14·5	0·78	0·05
16	10·0	0·13	0·01
16	22·0	0·18	<0·01
(*c*) *Bladder mucosa and bone present.*			
18	1·0	0·41	0·41
18	0·8	0·099	0·12
18	3·1	6·80	0·22
18	0·8	0·198	0·25
26	1·3	0·20	0·16
26	1·0	0·13	0·13
26	1·5	0·18	0·13
26	2·7	0·57	0·21
26	1·6	0·25	0·16

Contrasting sharply with these index values in unossified tissues and controls, the 18- and 26-day tissues containing bone (Table II *c*) without exception contained large amounts of phosphatase; the *A/W* value varied from 0·12 to a maximum of 0·41. These values are of the same order of magnitude as those obtained by Fell and Robison [1929, 1930] for normal and embryonic ossifying cartilage and bone. The variations can be accounted for in part by the different amounts of soft tissues in relation to bone in the material examined. In the presence of bone, therefore, phosphatase was present in amounts 2·4 to 8 times the maximum present in the highest of non-ossified tissues and 10 to 20 times the mean values for these tissues (Table III).

Table III. *Summary of ratios of phosphatase to dry weight A/W.*

	Controls		Transplantation experiment		
	Bladder mucosa	Rectus sheath	Rectus sheath adjacent to transplant	Rectus sheath bladder epithelium. No bone	Rectus sheath bladder epithelium. Bone
Maximum	0·03	0	0·05	0·05	0·41
Minimum	<0·01	0	0	0	0·12
Mean	0·02	0	0·01	0·02	0·20

SUMMARY.

The histological and chemical relationships of the enzyme (phosphatase) effecting hydrolysis of certain phosphoric esters were studied in the various stages of heterotopic osteogenesis in transplants of the urinary bladder epithelium to the abdominal fibrous tissue in the dog. In the presence of bone so formed the ratio of phosphatase activity to dry weight was very high, at least 2·5 to 8 times higher than the maximum, and 10 to 20 times the mean values obtained for the control tissues without bone.

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LXXXI. FAT METABOLISM IN MUSCULAR EXERCISE.

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A rise in the blood-fat during muscular exercise was first described by Patterson [1927], whose experiments left no doubt as to the reality of the phenomenon. His suggestion that the rise in blood-fat is a response to the demand of the working muscles for fat agrees with the observation of Lafon [1913] that the blood leaving a working muscle contains less fat than the blood entering it. The phenomenon of an alteration in the lipoid content of the blood during muscular exercise appears to be of importance not only from the point of view of blood chemistry, but also on account of the controversy which has arisen as to whether muscle is capable of utilising fat as a source of energy. The experiments described in this paper have been undertaken to extend the work of Patterson by elucidating the source and the nature of the extra fat which appears in the blood, the cause of its appearance, the chemistry of muscle with respect to the use of fat, and the relationship between the rate of utilisation of fat and its concentration in the blood.

Experimental.

Preparation of the subject. The subjects, with one or two exceptions noted later, were healthy young men, used to taking moderate exercise, and were on a normal mixed diet. At the time of the experiment they had fasted for 13–15 hours. They had either spent the night in the laboratory, or had rested there in bed since early morning. In either case, a state as nearly basal as possible was assured at the commencement of the experiment. For those experiments involving measurements of the gaseous exchange, this condition was obviously essential, since it provided the only possible base line from which to measure the excess metabolism during the exercise. Further, preliminary experiments showed that any previous activity on the part of the subject considerably modified the effect of a given amount of muscular work on the level of the blood-fat. These precautions were not observed by Patterson in all of his experiments, which doubtless explains some of the quantitative discrepancies between his results and those about to be described.

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Mode of exercise. The subjects were exercised on a bicycle ergometer at a steady rate. To most of the subjects cycling was an unaccustomed form of exercise, and it was found impossible for them to work for long at a greater rate than about 1100 kg. m. per minute. Short periods of work such as were employed by Patterson in his running experiments were not feasible, since amount rather than rate of work seemed to be the important factor. To avoid a training effect the same subject was not used for more than one experiment except after the lapse of a considerable period.

Experiments involving measurement of respiratory exchange. After the subject had reached basal conditions, and had emptied his bladder, his expired air was collected in a 200 litre spirometer for a period of 25 minutes. A sample of blood was drawn and the bladder was again emptied. The work was then begun, and at intervals during its progress the expired air was collected for a period of 1 minute, the subject being connected to the spirometer for this purpose without interruption of the steady state of work. A number of preliminary experiments satisfied us that extrapolation from the results of these separate collections gave nearly as accurate a figure for the gaseous exchange over the whole period as the much more cumbersome and uncomfortable procedure of collecting all the expired air throughout the exercise. The oxygen consumption reached a steady value about 5 minutes from the commencement of exercise, and it was therefore necessary only to confirm this in each case by taking two samples during the latter part of the exercise with, in addition, one sample at the second or third minute of exercise. Special care was needed at the end of the exercise on account of the sudden alteration in the respiratory exchange at the beginning of recovery. On the completion of the work, the subject immediately lay down and was once more connected to the spirometer. A second sample of blood was then taken. Collection of the expired air was continued until the subject had again reached a basal condition, as shown by pulse and respiration rate, and minute volume of expired air. At the end of this period—usually an hour was allowed—the subject again emptied his bladder and a third sample of blood was procured.

Experiments not involving measurement of respiratory exchange. In two series of experiments it was not feasible to measure the respiratory exchange. The first of these was undertaken in order to determine the effect of a short period of work, following immediately after apparent recovery from one of sufficient magnitude to produce a rise in the blood-fat. In one or two of these the respiratory exchange was determined over the first part of the experiment, but it was found that the discomfort of the mask, worn over such a long period of time, precluded the proper return of the subject to basal conditions after the second period of exercise. In these experiments blood was drawn before and after each period of exercise. The second series had as its object the determination of changes in the blood during the progress of exercise, and blood samples were withdrawn, not only before and after, but at intervals during the performance of work. This procedure necessitated

interruption of the exercise, and, though the withdrawal of samples was performed as expeditiously as possible, the interruption rendered fallacious any measurement of the respiratory exchange with the means at our disposal.

Chemical methods. The respiratory exchange was calculated in the usual way by analysis of samples removed from the spirometer.

The urine was analysed for total nitrogen and urea-nitrogen in order to determine whether any of the extra metabolism during exercise and recovery was due to protein breakdown. It may be said at once that no increase in the rate of nitrogen excretion was found during the experiments (Table I).

Table I.

Subject	Nitrogen excreted during exercise and recovery g.	Nitrogen excreted during same time under basal conditions g.
R.G.	1·23	1·36
D.M.D.	0·80	0·80
C.P.S.	0·79	1·03
J.B.R.	1·28	1·05
D.S.	1·29	1·25
R.E.I.	1·08	1·18
De V.	0·75	0·84
W.McL.	0·35	0·49

Usually, in fact, the urine secreted during the period of exercise and recovery contained rather less nitrogen than that for the corresponding basal period. This slight decrease, no doubt, is to be attributed to an increased elimination of nitrogen by the skin.

In the various blood samples, total fat, soap, total cholesterol, lipoid phosphorus, the iodine number of the fatty acids, and the carbon dioxide-combining power were estimated.

The method used to estimate the total fat in blood was a modification of that of Stewart and White [1925]. This method has been criticised by Stoddart and Drury [1929]. In the work reported here no difficulty was found in obtaining duplicate analyses agreeing to within 5 % by using the technique of Stewart and White, and several other workers in this laboratory have also used this method with good results. It has long been realised, however, that this method of analysis estimates the phosphoric acid from the phospholipins as well as the fatty acids obtained from the fats and phospholipins by hydrolysis. To overcome this difficulty, a modification of the original technique was devised, involving filtration—the same device as was introduced by Stoddart and Drury [1929]. As before, the total lipoids were extracted from blood with alcohol-ether mixture, and were saponified by evaporation almost to dryness with 5 cc. of $N/10$ sodium hydroxide. Whereas in the original method, this alkali was exactly neutralised by adding 5 cc. of $N/10$ hydrochloric acid, a slight excess of acid was now added, with thorough shaking to aid liberation of the free fatty acids; the mixture was warmed to produce coagulation of the fine particles, and filtered through a fat-free filter-paper. The precipitated fatty acids were washed three times with 1 cc. of 5 % sodium chloride. The precipitate was then dissolved in alcohol and the solution was made up to 10 cc. 1 cc. of this solution was titrated with $N/10$ sodium hydroxide, using the Rehberg burette. From the mean of three titrations, the blood-fat was calculated as tripalmitin.

The use of this modification gave results which were consistently lower than those given by the original Stewart and White method. If the phosphoric acid of the phospholipins of the blood were titrating as a monobasic acid, the difference in the results given by the two methods would

be accounted for. Table II shows the results of analyses of bloods in which fat was estimated by both methods, and the lipoid phosphorus was also determined. It shows how closely the difference in the fat content as determined by the two methods agrees with the fatty acid which would correspond to the lipoid phosphorus if it were titrating as a monobasic acid.

Table II.

Blood sample	Fat (mg./100 cc.)		Lipoid phosphorus (mg./100 cc.)	
	Original method	Modified method	Observed	Calculated
1	654, 657	501, 521	15·0	16·0
2	659, 679	444, 454	20·1	24·0
3	426, 429	331, 338	12·3	10·4
4	461, 491	320, 350	16·4	16·0
5	659, 660	446, 442	22·5	24·0

The method of Stewart and White [1929] was used for the estimation of soaps, that of Myers and Wardell [1918] for cholesterol, that of McClure and Huntsinger [1928] for iodine values, that of Van Slyke and Neill [1924] for carbon dioxide-combining power, and that of Briggs [1924] for lipoid phosphorus.

Results and discussion.

The utilisation of fat by muscle. The use of the R.Q. for determining the nature of the fuel used during muscular exercise demands as a primary condition that the body shall be in exactly the same condition at the beginning and end of the measured period except for the loss of fuel. The probability is that this condition, except perhaps in very mild exercise of short duration, is never entirely fulfilled. Not only must the oxygen consumption and the R.Q. have returned to their original levels—which involves the inclusion of a recovery period—but so must the lactic acid, carbon dioxide content and combining power of the blood. Even so, the conditions before and after exercise may not be strictly comparable, since even moderate exercise involves the excretion of lactic acid, and it is not known how this excretion affects the urinary content of bicarbonate ions or the ammonia/urea ratio. Such factors as these, however, though they may introduce considerable errors in experiments of short duration where the gas volumes dealt with are small, cannot have a very great effect on the longer experiments reported here. The particular ones mentioned above, in fact, would tend to make the observed R.Q. higher than the actual one. Hence though they, and others of the same kind, may afford at any rate a partial explanation of the very high respiratory quotients observed by Best, Furasawa and Ridout [1928], in violent exercise of very short duration, they do not invalidate the deductions drawn here.

In Fig. 1 the amount of work performed by a number of subjects is plotted against the R.Q. for exercise and recovery at the end of which the oxygen consumption, R.Q., and carbon dioxide-combining power of the blood had returned to normal. The figures used in constructing this curve were, of course, those for the extra metabolism due to the exercise, the basal metabolism having been deducted from the gross figures.

In three cases the R.Q. for small amounts of work was very much lower

than was expected, a result which indicates, if the conclusions drawn later are justified, a much earlier usage of fat. In these cases there appears to be either a relative inability to utilise glycogen in the normal way or a very low store of glycogen, and it is significant that all three are cases of diabetes mellitus. Since the evidence in the literature [Lusk, 1928] indicates that in

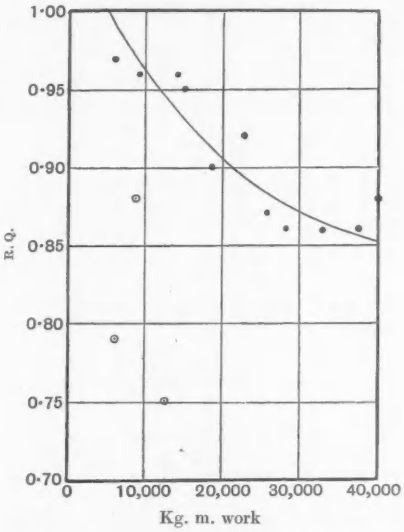

Fig. 1. Relationship between the amount of work and the R.Q. for exercise and recovery.

▪ Normal. ⊙ Diabetic.

this condition the muscle-glycogen is maintained at the usual level long after the remaining stores are depleted, and since the cases reported here were under control, it is reasonable to conclude that the fault lay in the utilisation of carbohydrate rather than in its lack. It is significant that glycosuria was not produced by the exercise.

With small amounts of work (4000–5000 kg. m.), the excess R.Q. was at or very near 1·0 (in two cases values appreciably above this were obtained), but with increasing amounts of work, the R.Q. for exercise and recovery steadily fell. In spite of the fact that twelve individuals are represented in the curve, the proportionality between the amount of work performed and the R.Q. is definitely marked, and goes far to support the substantial truth of the figures. It is perhaps advisable to reiterate here that the rate at which the different subjects performed the work was nearly constant, the tension on the ergometer bicycle and the rate of pedalling being kept as nearly constant as possible. The conclusion seems justified that, although the muscles prefer carbohydrate as a fuel, they are fairly rapidly forced to fall back on some other

fuel of lower R.Q. In no case did the results of urine analysis indicate any usage of protein.

There remains fat, which, therefore, one is forced to suppose is used by the muscles as a secondary source of energy. The alternative hypothesis that extra fat is used in the basal processes, thereby liberating carbohydrate for use in the working muscles, is disproved by a consideration of the amounts involved. Thus in one experiment, with R.Q. 0·96 for exercise and recovery, the relevant data are as follows. This experiment has been chosen for illustrative purposes since, with the small amount of fat utilisation involved, it offers a less convincing example than those experiments with lower R.Q.

R.Q. for exercise and recovery	0·96
Oxygen consumption for exercise and recovery	24·17 litres
Duration of exercise and recovery	85 minutes
Calculated fat used during exercise and recovery	3·31 g.
Heat equivalent of fat used	31·35 cals.
Basal oxygen consumption for 85 minutes	26·28 litres
Basal R.Q.	0·77
Nitrogen excretion during 85 minutes	1·097 g.
Carbohydrate used in basal metabolism during 85 minutes ...	3·55 g.
Heat equivalent of carbohydrate	14·86 cals.

The amounts of fat and carbohydrate are calculated from the tables given by Lusk [1928]. It is evident that the energy available from the whole of the carbohydrate used for basal metabolism during the period of exercise and recovery would not, if entirely diverted to the working muscles, obviate the necessity of their calling upon fat.

The R.Q., though it allows the deduction that fat is used as a fuel by the working muscles, gives no information as to whether the fat is used directly or only by conversion to carbohydrate.

The controversy as to which of these alternative routes is followed by the fat used in muscular exercise hinges largely on the problem of the relative efficiency of fat and carbohydrate.

It is rightly argued that if fat is utilised by direct oxidation, muscular efficiency should be unaltered when it replaces carbohydrate; on the other hand, utilisation only of carbohydrate derived from fat necessarily implies a loss of energy, and therefore a decreased efficiency. Moreover, the loss of efficiency should be the same for every individual. In considering the magnitude of such a hypothetical loss of efficiency, one is faced with the difficulty that the conversion of fat to carbohydrate in the animal body has never been conclusively proved, and that those workers whose experiments have seemed to demonstrate the possibility of such a conversion have thrown no light on the mechanism. The smallest wastage of energy, as has often been pointed out, would occur in the unlikely event of conversion of the whole of the carbon of the fat molecule into carbohydrate, and even then some 25 % of the total available energy would be lost.

The classical work of Krogh and Lindhard [1920], in which the maximum

loss of efficiency was found to be only 11 %, is often cited as disproving the fat to carbohydrate conversion hypothesis. The original authors, however, are more cautious, and merely state that their experiments cannot be used as proof that fat is necessarily converted to carbohydrate before being utilised by working muscle.

The change over from carbohydrate to fat as muscular fuel is, of course, not sudden and complete. Even when fat has become the preponderating source of energy, carbohydrate is still being used though in diminishing amount. This is shown in Fig. 2, in which the amounts of fat and carbohydrate used for various amounts of work (and recovery) are plotted against the amounts of external work performed. The figures for the amounts of fat and carbohydrate used are calculated in the usual way from the observed excess oxygen consumption and R.Q. It is evident from the curves that as the amount of work increases the amount of fat used increases, but so also does the amount of carbohydrate. Even with R.Q. 0·80, carbohydrate is still being used; the R.Q. is not simply the mean result of the early exclusive use of carbohydrate and the later exclusive use of fat.

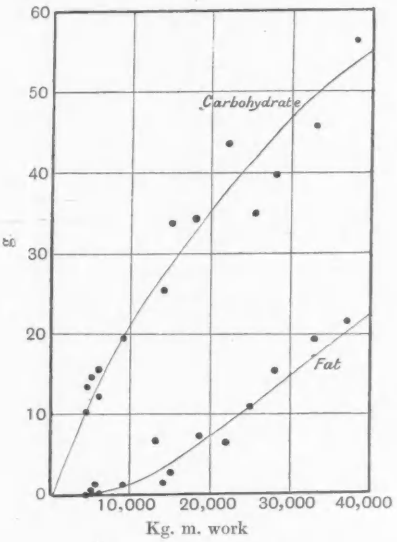

Fig. 2. Relationship between the amount of external work and the amounts of fat and carbohydrate oxidised.

The R.Q. for a complete period of work and recovery can never be that of fat alone—except possibly in the case of a complete diabetic, and then only on the assumption that the diabetic cannot oxidise carbohydrate or lactic acid. Hence the loss of efficiency to be expected is always less, and, with R.Q. 0·80

and over, very much less than the calculated 25 %. Thus, in one of the experiments made by the present authors, the R.Q. and oxygen consumption for work and recovery showed the utilisation of 56·8 g. of carbohydrate and 21·56 g. of fat, with R.Q. 0·86. A simple calculation shows the expected loss of efficiency to be only 11·5 %.

In the experiments of the present authors no loss of efficiency was observed when work was performed at the expense of fat (Table III). It is admitted that the experiments were not specially designed for the purpose of showing such change in efficiency, and that in no case was a series of experiments undertaken upon the same individual with varying amounts of work and at varying R.Q. Nevertheless, since the conversion of fat into carbohydrate involves a definite wastage of energy irrespective of individuals, it seems fair to suppose that any such wastage would be shown in the average results from a large number of subjects. Since the subjects were not trained cyclists, the fact that only one experiment was performed upon each was an actual advantage, for it eliminated any fallacy due to training. The numbers reported here are not, it is true, large, but, as they show no suggestion of a wastage of energy with the lower R.Q., and as the distribution in each series is nearly the same, it has been thought worth while to report them (Table III).

Table III.

R.Q.	1·0	0·95–0·99	0·90–0·94	0·85–0·89	0·80–0·84
Lowest	4·76	4·23	4·32	4·11	4·06
Highest	5·71	5·15	5·38	5·65	5·70
Mean (no. of exps)	5·12 (6)	4·82 (4)	4·86 (6)	4·78 (7)	4·60 (6)

They differ radically, of course, from those of Krogh and Lindhard [1920], and it seems possible that the difference may lie in the fact that all the subjects dealt with here were on an ordinary mixed diet, while Krogh and Lindhard used high and low carbohydrate diets to obtain the variations in R.Q. Moreover, the rate of work in the experiments reported here was much greater than in those of Krogh and Lindhard.

A wastage of energy in the utilisation of fat should become apparent not only in the calculated efficiency, but also in another way. The wastage, if fat is converted to carbohydrate, is due to some such process as loss of hydrogen which is oxidised to water. Thus, in the most favourable conversion,

$$\underset{\text{Glyceryl oleo-stearate}}{2C_{57}H_{106}O_6} + 47O_2 = 19C_6H_{10}O_5 + 11H_2O.$$

The utilisation by the muscles of 1 g. of glycogen derived from fat demands, according to this equation, the consumption of 1·099 litres of oxygen instead of the 0·828 litre required by the glycogen itself—an oxygen wastage of 31·5 %. On the other hand, the direct utilisation of fat involves no loss of oxygen, and the curve showing oxygen consumption per calorie equivalent of work done is simply that of the "calorific value" of oxygen calculated by Zuntz and Schumburg [1901]. These two curves for the consumption of oxygen

per calorie equivalent of work are shown in Fig. 3. In these curves allowance is made for the simultaneous use of carbohydrate, the relative amounts of the two fuels being calculated on the basis of Fig. 1. The experimentally determined values are also shown in Fig. 3, and, though there is a certain amount of scattering due to the employment of many different individuals, it is evident that by far the closer approximation is given by the curve corresponding to direct utilisation of fat.

In the curve, as in the table of efficiencies, are included the results from the three diabetic subjects, and it is interesting to note that in spite of their

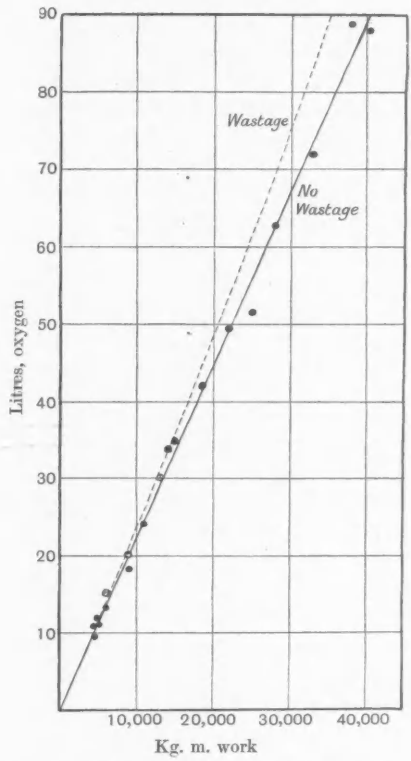

Fig. 3. Relationship between the amount of work and the oxygen consumption for work and recovery. The continuous line represents the volume of oxygen expected if there is no wastage consequent upon the utilisation of fat. The dotted line represents the oxygen consumption calculated on the basis of a 30 % wastage for the conversion of fat to carbohydrate.

▪ Normal. ⊙ Diabetic.

abnormally low R.Q., indicating the utilisation of an abnormally high proportion of fat, they show no deviation from the normal efficiency.

Though no finality is, of course, claimed for these results, they tend very

definitely to oppose the view of the necessary conversion of fat to carbohydrate prior to its utilisation by the working muscles, and to support the view that fat and glycogen are alternative fuels although the latter appears to be used preferentially.

The theoretical need for a conversion of fat to carbohydrate has, indeed, been greatly weakened by the recent work of Eggleton and Eggleton [1927] on creatinephosphoric acid and of Lundsgaard [1930] on muscle poisoned with iodoacetic acid, which appears to show that the breakdown of glycogen to lactic acid can no longer be regarded as an essential in muscular contraction. Even if the glycogen-lactic acid cycle be considered essential, it may well be that fat, by its direct oxidation, is capable of sparing lactic acid, leaving more for resynthesis to glycogen and so conserving the supplies of carbohydrate.

It is perhaps worth pointing out, though with diffidence in view of modern ideas as to the function of insulin, that if it be admitted that diabetes mellitus involves a failure to oxidise carbohydrate, calculations from cases of nearly complete diabetes show that such diabetics do not convert fat to carbohydrate. Such a conclusion follows, *e.g.* from the data in the case reported by Geyelin and Du Bois [1916], in which the observed respiratory exchange and heat production can only be explained in this way. The diabetic can oxidise fat as far as acetoacetic acid, and it is therefore a fair conclusion that any conversion of fat to carbohydrate is possible only after this stage, *e.g.* by oxidation to acetaldehyde followed by aldol condensation. Such a conversion would involve a wastage, not of 25 %, but of 80 % of the energy of fat, and would render quite impossible of accomplishment the amounts of work actually observed.

The variation in blood-fat in muscular exercise. The results of blood analysis (Table IV) show that the majority of normal subjects do experience some increase in the blood-fat, when, under fasting conditions, they perform work in excess of approximately 8000 to 10,000 kg. m. This figure is only approximate, since the exact point at which the rise in the blood-fat occurs depends to a considerable extent on the rate at which the work is performed. Table IV gives typical results of blood analyses. The experiments on normal subjects (*A*) show that the rise in blood-fat does not occur in every case (*e.g.* C.P.S., D.S.); when it does occur, it is transient, and has disappeared at the end of the recovery period.

Patterson [1926] observed that diabetic subjects almost invariably showed a fall in the blood-fat, even with the relatively small amounts of work they were able to perform. All his diabetic subjects, however, had initially an abnormally high blood-fat, a state of affairs which is usually present in this disease. The experiments summarised in Table IV *B* show that this fall in the blood-fat produced by exercise is characteristic, not of diabetes mellitus, but of the incidental high blood-fat level. It occurred in subjects whose fasting blood-fat was abnormally high, but who were apparently healthy in all other respects. Moreover, in two diabetic cases (Table IV *C*), with the basal blood-

Table IV.

	Subject	Work kg. m.	Blood sample	Fat mg./100 cc.	Cholesterol mg./100 cc.	Lipoid P mg./100 cc.	Soap mg./100 cc.
A	R.G.	5,500	*a*	492	134	—	40·0
			b	433	139	—	37·9
			c	481	128	—	41·2
			d	579	133	—	41·2
	C.P.S.	14,966	*a*	689	136	21·7	27·9
			b	693	136	21·9	17·6
			c	522	137	21·6	24·6
			d	562	137	20·0	27·2
	D.S.	18,564	*a*	478	143	20·7	—
			b	453	144	22·3	—
			c	450	125	23·1	—
			d	481	127	20·3	—
	R.E.I.	19,123	*a*	607	118	13·3	30·7
			b	791	114	12·1	30·2
			d	583	118	11·6	31·2
	R.G.	28,050	*a*	480	145	—	30·7
			b	588	144	—	45·7
			d	434	146	—	39·8
	R.E.I.	33,000	*a*	511	118	18·8	23·9
			b	674	118	16·5	25·2
			c	467	—	19·0	25·0
			d	425	—	15·0	23·9
	De V.	37,150	*a*	376	120	15·3	23·6
			b	511	142	15·2	24·6
			c	464	127	17·2	24·1
			d	460	122	17·5	24·5
B	D.M.D.	4,300	*a*	853	208	21·7	28·2
			b	685	209	21·9	26·7
			c	522	208	21·8	22·6
			d	562	207	21·7	27·2
	J.B.R.	14,000	*a*	1103	139	15·5	29·7
			b	831	141	18·3	35·7
			c	694	140	15·1	42·7
			d	899	139	14·5	42·7
C	J.McC.	8,600	*a*	770	—	—	—
			b	825	—	—	—
	W.McL.	12,300	*a*	690	—	—	—
			b	812	—	—	—
D	F.P.C.	22,250	*a*	310	119	15·9	36·0
			b	660	121	15·5	34·7
			c	510	121	—	35·7
			d	410	120	15·6	35·2

a = Before work.
b = After work.
c = After partial recovery.
d = After complete recovery.

fat not much above the normal level, exercise produced a small but definite increase as easily as in normal subjects. Nor, probably, is it mere coincidence that in one case the converse appeared to hold, and that a healthy subject with an abnormally low fasting blood-fat level showed an abnormally great increase after a moderate amount of work (Table IV *D*). These results lead to the conclusion that the blood-fat content tends to reach the normal level before it shows the increase usually given by healthy individuals. This preliminary change takes place under the influence of moderate work, definitely

below the amount required to produce a change in the blood-fat content of subjects whose basal blood-fat is within the normal range.

Those experiments in which the subject performed a second period of work after recovery from the first (Table V) demonstrate that a rise in the blood-fat is rather more easily obtained during the second period than during the first. This point is of importance as emphasising the necessity of obtaining basal conditions before starting the exercise if comparable results are to be obtained from a series of experiments on different individuals. It also tends to support the contention that the increase in blood-fat during exercise is in some way connected with the utilisation of fat by the muscles. There can be no doubt that at the commencement of the second period of exercise the body stores of glycogen have been somewhat depleted, and hence the muscles—if they can use fat—must call for fat earlier, though only slightly so, than in the first period.

Table V.

Subject	Blood sample	Fat mg./100 cc.	Cholesterol mg./100 cc.	Lipoid phosphorus mg./100 cc.
R.G.	Basal	611	145	14·6
	After 1st work (28,000 kg. m.)	719	144	14·2
	After recovery	565	146	14·6
	After 2nd work (11,000 kg. m.)	619	147	14·3
R.E.I.	Basal	607	118	13·3
	After 1st work (19,000 kg. m.)	791	114	13·1
	After recovery	583	118	11·6
	After 2nd work (9000 kg. m.)	647	118	13·7

The results so far described confirm the statement that muscular exercise is accompanied by an increase in the blood-fat, though they show that this is not an invariable result. It was desired, however, to find in what constituents of the total blood-fat the increase occurred. The analyses (Tables IV and VI) showed that the increase lay solely in the triglyceride fraction, which, according to Channon and Collinson [1929], forms a very small part of the total blood-lipoids under resting conditions. While the total fatty acids increased, the lipoid phosphorus and the cholesterol showed either no change or only small and inconstant variations. The free fatty acid (soap) content of the blood remained unaltered, and though there was possibly an increase in the amount of esterified cholesterol, any such increase was quite incapable of accounting for the observed increase in the total fatty acid. Figures in support

Table VI.

Subject	Total cholesterol mg./100 cc.	Fat required to esterify completely mg./100 cc.	Rise in fat mg./100 cc.
R.E.I.	118	13·7	163
FF.P.C.	121	14·0	350
De V.	142	16·5	135
R.G.	144	16·7	108
R.E.I.	114	13·2	184
R.M.L.	136	15·8	92
R.G.	129	15·0	220

of this statement are given in Table VI, which is based on the ratio of esterified to unesterified cholesterol given by Channon and Collinson [1929].

Finally, since Patterson's figures were obtained by the use of the original Stewart and White method, it is important to note that exactly similar results are obtained—as in the experiments here described—by use of the modified method which estimates only fatty acids and does not include phosphoric acid. Otherwise the apparent increase in the blood-fat during exercise might conceivably have been regarded as due merely to an altered state of dissociation of the phospholipins.

The iodine value of the blood-fat before and after exercise was determined in a number of cases (Table VII), and although it was found to be very variable for different subjects, it altered only very little in the same subject as a result of exercise, and in no case did it show a rise. This suggests very strongly that the source of the fat poured into the blood stream in response to stimulation by exercise is the adipose tissue and not the liver.

Table VII.

Subject	Work	Fat mg./100 cc.	Iodine value	Subject	Work	Fat mg./100 cc.	Iodine value
T.F.	Basal	426	63	E.B.H.	Basal	364	26
	11,550 kg. m.	421	65		13,250 kg. m.	374	24
	16,885	661	52		20,500	485	14
	24,650	448	32		26,650	387	26
E.G.	Basal	660	84	A.T.B.	Basal	444	22
	12,750 kg. m.	690	65		10,250 kg. m.	454	27
	18,965	865	62		16,250	454	34
	28,000	757	78		23,050	444	36

The variation of the blood-fat during muscular exercise. Those experiments in which blood-fat estimations were made during the performance of work showed a curious result which ultimately shed light on the marked individual variations and apparently haphazard responses obtained in previous experiments. It appeared that, instead of the blood-fat steadily increasing during exercise, it first rose and then fell. This result was obtained in a considerable number of individuals, and typical cases are shown in Fig. 4. When the results were plotted together as a composite curve, it appeared that actually there was a rise in the blood-fat, followed by a fall and then by a second rise (Fig. 5, dotted line). Moreover, the results obtained from nearly all the previous experiments could be fitted to this curve. Certain exceptions were noted, as when the rate of work differed markedly from that usually employed, or when the subject had an abnormal resting blood-fat.

The shape of the composite curve had been disguised in the later individual experiments shown in Fig. 4 by the fact that only three points were obtained. To test whether the exceptions to this composite curve were really due to the one observed variation in experimental technique—the rate of work—a second series was undertaken in which the subjects worked at a rate of 1100 kg. m. per minute instead of the 900 kg. m. per minute which had

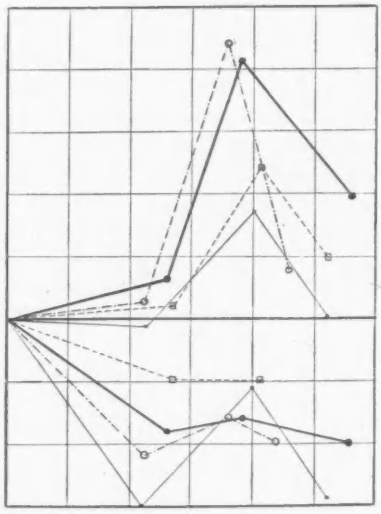

Fig. 4. The blood-fat and carbon dioxide-combining power during exercise. Individual cases. (Ordinates as for Fig. 5.)

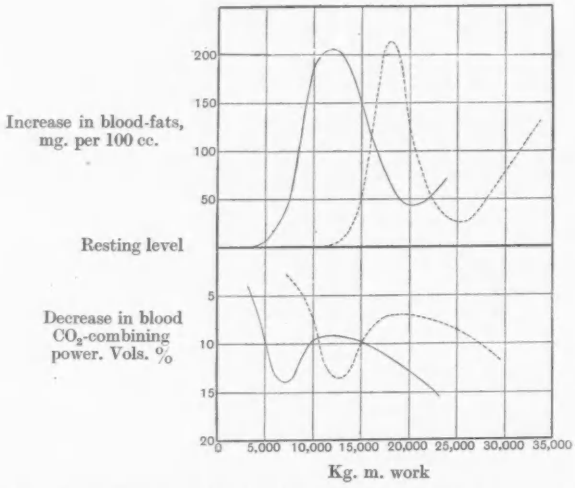

Fig. 5. Composite curves showing changes in the blood-fat concentration and the blood carbon dioxide-combining power during exercise. The dotted line represents changes at 900 kg. m. per minute, the continuous line at 1100 kg. m. per minute.

previously been the rule. The results of blood-fat determination from this series fell on a second composite curve (the continuous line in Fig. 5) of exactly the same shape as the first, but showing the changes in blood-fat concentration to take place after a smaller amount of work than previously. These variations in the blood-fat during work were entirely unexpected, and could not be correlated with a steadily increasing consumption of fat by the working muscles. Yet experiment had shown that such a steadily increasing consumption did indeed exist (Fig. 2).

It became necessary, therefore, to discard the simple suggestion that the increase in blood-fat during exercise was a direct response to a demand by the muscles for an alternative fuel consequent upon the utilisation of glycogen. Attention was thus directed to the discovery of some stimulus which could act as a connecting link between the two phenomena. The production of lactic acid had suggested itself as a possible causative factor in the rise in blood-fat, which, at that time, was all that had been observed. For ease of measurement the carbon dioxide-combining power of the blood had been determined in all samples. The results of these analyses showed at first somewhat irregular decreases in the carbon dioxide-combining power of the blood following exercise, but it was only when blood samples were analysed during the course of exercise that any real relationship between the blood-fat and the carbon dioxide-combining power was observed. It then transpired that the carbon dioxide-combining power, instead of falling steadily with increasing amounts of work to the acidosis level, followed a course which was roughly the reciprocal of that followed by the blood-fat (see Fig. 4). A composite curve of all the results obtained, just as in the case of the blood-fats, was sinuous, showing a fall followed by a partial recovery and only then a steady progression to the acidosis level.

The second series of experiments in which blood samples were drawn during the progress of exercise showed a composite curve of exactly the same character as the first. Again, however, as in the case of the blood-fat concentration, the fall occurred at an earlier period of the work. Moreover, in both series, the fall in the carbon dioxide-combining power preceded the rise in blood-fat, and its partial recovery preceded the fall in blood-fat. Whether this time relation between the curve of carbon dioxide-combining power and that of the blood-fat concentration has any real significance, it is too early to say definitely. It is none the less interesting to speculate whether it indicates a causative relationship between the two phenomena.

SUMMARY.

1. When normal healthy men performed muscular work on an ergometer bicycle at rates varying from about 800 to 1200 kg. m. per minute, the blood-fat usually rose after about 8000 kg. m. of work had been done.

2. The increase in the blood-fat concentration appeared earlier with greater rates of work.

3. After recovery from a first period of work, a second period produced a rise in the blood-fat more easily than usual.

4. A high fasting blood-fat in normal or diabetic subjects led to a preliminary fall when work was performed; an abnormally low blood-fat was increased by relatively small amounts of work.

5. This alteration in the blood-fat was confined to the glyceride fraction, and the extra fat was probably derived from the adipose tissue.

6. Continuance of work led to a return of the blood-fat towards normal (at about 18,000 to 21,000 kg. m.), and later to a second rise.

7. The carbon dioxide-combining power of the blood during muscular work of this type followed a course which was roughly the reciprocal of the blood-fat, but the changes in the carbon dioxide-combining power preceded those in the blood-fat.

8. The R.Q. for exercise and recovery was unity for amounts of work up to about 5000 kg. m., and thereafter fell steadily with increasing amounts of work.

9. Analysis of the R.Q., oxygen consumption, and nitrogen excretion showed that protein was not used for work, that carbohydrate continued to be used throughout, though in decreasing amounts, and that fat was used in increasing amounts.

10. There was no relationship between the utilisation of fat and the changes in the blood-fat concentration.

11. No diminution in efficiency was observed at the lower R.Q., and the figures did not suggest any conversion of fat to carbohydrate.

12. Diabetic subjects, though performing work at a lower R.Q. than normals, showed no less efficiency, and no wastage of oxygen.

13. A modification of the Stewart and White [1925] method for the estimation of blood-fat is described.

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LXXXII. THE NATURE OF TYROSINASE.

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(*Received March 30th, 1931.*)

The view that tyrosinase is a mixture of a number of components has been held by several investigators. Haehn [1920] reported that potato tyrosinase lost its activity on dialysis or ultrafiltration, but regained it on addition of the ultrafiltrate or boiled juice. He concluded that the activator was inorganic in nature. Raper and Wormall [1923], while partially confirming Haehn's finding, noticed that the boiled juice of new but not of old potatoes had an accelerating effect. They also adduced evidence to show that the activator in potato juice is organic in nature.

In an earlier paper [Narayanamurti and Ramaswami, 1929] it was shown that on ultrafiltration of *Dolichos* tyrosinase the residual liquid on the ultrafilter was active and that the addition of the ultrafiltrates to the residual liquid did not cause any acceleration. The following additional results have so far been obtained.

Experimental.

In this investigation the enzyme prepared by three different methods, *viz.* (*a*) ordinary dialysis, (*b*) ordinary dialysis followed by ultrafiltration, and (*c*) ordinary dialysis followed by precipitation with alcohol, has been studied.

Preparation of dialysed extracts. About 200 g. of the finely ground meal of the ungerminated seed of *Dolichos lab lab* were extracted with 600 cc. of distilled water in glass-stoppered bottles at room temperature for 3 days in presence of toluene. After filtering through paper pulp the extract (containing a large quantity of protein) was dialysed in large parchment filters against flowing distilled water for 1 week, when all the protein was precipitated. The solution was then centrifuged to remove suspended matter, the resulting liquid being clear.

Purification by repeated precipitation with alcohol. Dialysed extracts were precipitated with 95 % alcohol, the flocculent precipitate being allowed to settle in an ice-chest and separated by decantation and filtration. The residue on the filter was ground up with distilled water and filtered, alcohol again being added to the filtrate. The whole of the first precipitate does not dissolve and the undissolved residue possesses some activity. The precipitated enzyme is very small in quantity and is dried *in vacuo*.

Ultrafiltration. The ultrafiltration apparatus used was an improved form of that described by Brukner [1926]. About 200 cc. of the dialysed extract were filtered through parchment paper at a pressure of 50 kg. The solid residue was removed, ground up with distilled water and filtered through ordinary filter-paper. This filtrate was again subjected to ultrafiltration, the whole process being repeated a third time, the ultrafiltrates being collected each time. The residue finally obtained was dried in a vacuum desiccator.

Effect of addition of ultrafiltrate on the activity of the enzyme. The effect of addition of the ultrafiltrate on the activity of the enzyme was investigated at 30° and the results are given in Table I, the activity being expressed in cc. thiosulphate.

Table I.

80 cc. of 0·06 % tyrosine solution and 20 cc. of acetate buffer at p_H 6·5 and 10 cc. of toluene were taken in each case.

No.	Enzyme solution	Water or ultrafiltrate	Time (hrs.) 2	4	5
I	20 cc. dialysed extract	10 cc. water	4·5	5·2	6·3
II	20 cc. of a solution of ultrafiltered enzyme	,,	2·0	3·4	4·0
III	,,	10 cc. ultrafiltrate 1	1·6	1·9	3·3
IV	,,	10 cc. ultrafiltrate 2	1·4	2·9	3·5
V	,,	10 cc. ultrafiltrate 3	1·3	3·1	3·7
VI	,,	10 cc. boiled juice	1·8	3·4	4·1

It is clear that the enzyme has not lost its activity on ultrafiltration and that the addition of boiled juice or the ultrafiltrate does not cause any acceleration. On the other hand, slight inhibition is caused, this being greatest with ultrafiltrate 1 and least with 3. The solid content of enzyme solution I (dialysed) was 4 times that of the ultrafiltered enzyme solution but its activity was only 1·5 times as great. Results of experiments done at the same solid content are given in Table II.

Table II.

Time in hours	Activity in cc. thiosulphate: Ultrafiltered enzyme	Dialysed extract
1	1·0	0·6
2	1·6	—
4·5	3·3	1·5
7·0	4·5	2·0

The results clearly indicate that the ultrafiltered enzyme is more than twice as active as the dialysed preparation. Conductivity measurements showed that the conductivity of the ultrafiltered enzyme was one-twelfth that of the dialysed preparation. It is therefore evident that ultrafiltration is a good method of purification and that the enzyme is not separated into two components as claimed by Haehn.

Comparison of precipitated enzyme with the dialysed extract. The results are given in Table III.

Table III.

Time in hours	Activity in cc. thiosulphate	
	Precipitated enzyme	Dialysed extract
0·5	4·0	5·2
1·0	8·1	6·4
2·0	10·6	9·5

It is evident that the precipitated enzyme is slightly more active than the dialysed extract.

Electro-osmosis. It has been shown previously [Narayanamurti and Norris, 1928] that cholam malt diastase could be separated into two components by electro-osmosis. Similar experiments were tried with tyrosinase, but it was found that the addition of anode and cathode cell liquids to the middle cell liquid caused only a slight diminution of activity, thus again showing that tyrosinase cannot be separated into two components.

Summary.

Dolichos tyrosinase purified by ultrafiltration is more active than the dialysed preparation. Addition of ultrafiltrate causes no acceleration; on the other hand, slight inhibition is observed.

On repeated precipitation tyrosinase does not lose its activity.

Addition of cathode cell or anode cell liquid to the middle cell liquid of tyrosinase subjected to electro-osmosis in a five-celled apparatus does not cause any increase in activity. Slight inhibition is caused.

All these results clearly indicate that tyrosinase cannot be separated into two components. Any activator present must be in the colloidal condition or bound to a colloidal carrier.

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LXXXIII. THE ACTION OF TANNASE ON GALLOTANNIN.

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(*Received March 31st, 1931.*)

Tannase hydrolyses gallotannin with production of gallic acid. The activity of tannase can thus be measured by estimating either the unchanged gallotannin or the gallic acid formed. Micro-methods were therefore elaborated in this laboratory for the estimation of gallotannin [Spiers, 1914; Rhind and Smith, 1922] and gallic acid [Nicholson and Rhind, 1924]. The application of these methods to the tannase problem, using gallotannin freed from glucose [Nierenstein, Spiers and Hadley, 1925], gave an unexpected result, namely, that more gallotannin was hydrolysed than corresponded with the gallic acid produced. It is now shown that this is due to the presence of an enzyme in tannase which destroys gallic acid. This enzyme, for which the name pyrogallase is suggested, is also produced by growing *Aspergillus niger* on media which contain pyrogallol or gallic acid. The presence of pyrogallase in tannase obviously forbids the use of tannase in connection with the chemistry of gallotannin, as was originally planned by us, and this also applies to other tannins which yield gallic acid on hydrolysis [*cf.* Fischer and Bergmann, 1918; Freudenberg, 1919, 1920; Freudenberg and Peters, 1920; Perkin and Uyeda, 1922; Freudenberg and Blümmel, 1924; Freudenberg and Frank, 1927]. The production of pyrogallase by moulds thus affords an explanation of the fact, well-known in industry, that the action of moulds produces appreciably less gallic acid from gallotannin than does acid hydrolysis, although Pottevin [1901] claims a yield of 98 % of gallic acid by the action of *A. niger* on gallotannin.

Experimental.

I. *Cultivation of* A. niger. Czapek's medium as modified by Knudsen [1913] was used; the concentrations of gallotannin, pyrogallol and gallic acid being 4, 2·5 and 0·8 % respectively. The crops were grown in Fernbach flasks or in ordinary large bottles placed on their sides. Each flask held about 350 cc. of medium and each bottle about 150 cc. when exposing a maximum surface. They were plugged tightly with cotton wool, covered with leadfoil and sterilised by intermittent steaming for an hour on three consecutive days. They were then inoculated from the required stock with a sterilised platinum needle. After being well shaken to induce the spores to spread over the surface

of the medium the vessels were placed in an incubator at 23°. After about 4 days the surface was covered generally fairly evenly by a thick white felt, and spores were beginning to form. At this stage the felt was removed, as during the spore formation the fungus excretes enzymes freely into the medium [Dox, 1910]. The felt was removed with a glass rod, washed with tap-water to remove both spores and medium, squeezed as dry as possible, put into a flask and covered with acetone, in which it was allowed to stand overnight. This killed the mycelium, dissolved out the colouring matter and removed the final traces of medium. The following day it was ground up in a mortar with fresh acetone and filtered by suction, the process being repeated until the acetone washings were practically colourless and the mycelium reduced to a fine powder. This was finally washed in a little ether, and after being again filtered by suction was left exposed in a cupboard in an evaporating dish until no smell of ether could be detected. The resulting mouse-grey powder is referred to as "mycelium-powder." It was put into a stoppered bottle and kept in the dark until required.

In a few cases one medium was used to grow more than one crop, but between each crop it was filtered through glass wool and re-sterilised. It was found, however, that although the felt was generally greater in amount in the second crop than in the first, it was less reactive, and in the majority of cases, therefore, only one crop was grown on each medium.

II. *Observations on the behaviour of* A. niger *during spore formation.* Reference has already been made to the observation recorded by Dox that the fungus excretes enzymes during spore formation. Our results using the methods described by Dox are tabulated below. They confirm Dox in every respect.

Table I.

Enzymes other than tannase and pyrogallase	Substrate used for detection	Mycelium-powder	Cultural solution before sporulation	Cultural solution after sporulation
Oxidase	Guaiacum	−	−	−
Peroxidase	Guaiacol	−	−	−
Laccase	Pyrogallol	−	−	−
Tyrosinase	Tyrosine	−	−	−
Protease	Fibrin	?+	−	−
	Gelatin	−	−	−
	Egg-white	−	−	−
Emulsin	Salicin	+	−	−
	Amygdalin	+	−	+
Diastase	Starch	+ + +	−	+ +
Invertase	Sucrose	+ + +	−	+ +
Maltase	Maltose	+ +	−	+
Lactase	Lactose	+ +	−	+
Raffinase	Raffinose	+	−	+
Amidase	Urea	+	−	+
Hippurase	Hippuric acid	−	−	−
Lipase	Ethyl acetate	+ +	−	+

III. *Hyper-cultivation.* The term hyper-cultivation is used here to designate the growing of the fungus for several successive asexual generations on gallotannin, a freshly prepared medium being used for each generation.

The results obtained show that it is possible to increase the yield of tannase by this method, and that a physiological change is apparently brought ab which persists even after the fungus is removed from contact with gallotannin. Thus in working with three cultures of *A. niger* and growing each for six asexual generations on gallotannin, the following values, definitely showing an increased tannase production, have been obtained. Taking the activity of the first generation of strain A to be 100 after 24 hours' hydrolysis:

Table II.

Strain A		Strain B		Strain C	
Generation	Activity	Generation	Activity	Generation	Activity
1	100	1	254	1	247
2	113	2	300	2	449
3	88	3	161	3	269
4	167	4	184	4	198
5	137	5	216	5	328
6	155	6	264	6	420
	α 136		β 306		γ 435

In the case of all three cultures an inoculation was made from the fifth generation into a prune-agar medium (which was free from gallotannin). The new generation was again inoculated into a gallotannin-containing medium, and the crops thus produced have been labelled α, β and γ respectively. As will be seen, in each case they showed a greater activity than the first generation had done. This is of importance since it apparently indicates the persistence of the increased tannase production, probably due to a physiological change. As far as could be observed there was no morphological change.

IV. *Preparation of tannase and pyrogallase.* A suspension of 500 g. of mycelium-powder in 2 litres of distilled water containing a little chloroform is shaken for 200 hours and the solution filtered at the pump, the remaining solid being washed 4 times with 100 cc. of water containing a little chloroform. To the filtered solution are added 6 litres of absolute alcohol and the precipitate (40 g.) is collected after standing for 2–3 days. The solid, suspended in 150 cc. of distilled water containing a little chloroform, is shaken for 24 hours and filtered through thick filter-paper, and 2 litres of absolute alcohol are added to the filtrate. The precipitate formed (about 18 g. in the case of tannase and 15 g. in the case of pyrogallase) is collected and again purified by dissolving in 100 cc. of water, shaking for 24 hours, filtering and precipitating with 2 litres of absolute alcohol. In this manner the following average yields were obtained: 9 g. tannase, 6 g. pyrogallase and 2 g. pyrogallase by growing on media containing gallotannin, pyrogallol and gallic acid respectively.

V. *General properties of tannase and pyrogallase.* Both enzymes are completely soluble in cold water. Tested by Dox's methods they are found to give positive reactions for diastase, invertase, maltase, raffinase, amidase and lipase. The pyrogallol nucleus undergoes a profound change when acted on by pyrogallase, it loses its colour-producing properties with iron salts after

some time. The mechanism of this reaction is difficult to explain and all our attempts to isolate some definite disintegration product have led to disappointing results. Table III gives the values observed for the action of 0·1 g. of pyrogallase on 50 mg. of pyrogallol in 100 cc. of distilled water.

Table III.

Exp. 25.		Exp. 34.	
Time hrs.	Pyrogallol found mg.	Time hrs.	Pyrogallol found mg.
24	30·76	48	27·16
72	12·22	72	21·70
96	16·72	93	23·24
144	17·04	147	18·60
168	13·40	174	15·54
192	12·26	226	17·10
216	11·14	248	10·62
240	12·12	336	8·88
164	11·88	357	3·18

It must be noted that in these two experiments two different preparations of pyrogallase were used.

Table IV gives a typical experiment showing the amount of gallic acid produced by tannase from 100 mg. of anhydrous gallotannin. Column A records the amount of gallic acid found, column B the calculated amount of gallic acid from the estimated amount of the unchanged gallotannin.

Table IV.

Exp. 12.		
Time hrs.	A. Gallic acid found mg.	B. Gallic acid calculated mg.
0	1·59	—
24	13·93	19·10
48	19·74	24·80
72	26·16	31·60
144	37·46	40·70

SUMMARY.

It is shown that *Aspergillus niger* produces in addition to tannase an enzyme (pyrogallase) which destroys gallic acid.

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LXXXIV. THE CHEMICAL ASPECT OF THE DRYING OF TIMBER.

2. THE DRYING OF A SOFTWOOD.

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(*Received April 2nd, 1931.*)

In a previous communication [Campbell and Booth, 1930] an account was given of the chemical changes which accompany the drying of oak wood under various conditions. It was indicated that owing to certain well-defined differences in composition between hardwoods and softwoods these two classes of timber might be expected to react differently to similar conditions of drying. In pursuance of this indication the same experimental procedure which was applied to oak wood has now been applied to a softwood. The material used was the freshly felled mature wood of Silver fir in which there was no differentiation in colour between heartwood and sapwood. After conversion to sawdust the material was ground, as before, to pass an 80 mesh and be retained by a 100 mesh screen. The analytical methods were the same as those used in previous work, and the results throughout are expressed as percentages of the oven-dry weight of the original wood.

Experimental.

The moisture content of the 80–100 mesh material as determined by the standard oven-drying method was 34·58 % and this result was close to that obtained by the xylene method of Schwalbe [Schorger, 1926, pp. 505–506]. The drying experiments were carried out as follows.

(i) *Air-drying.* A weighed sample of the 80–100 mesh wood flour was placed in a glass jar covered with fine linen and left in the laboratory, with occasional mixing, to dry for a period of 200 days. The final moisture content was 13·27 %.

(ii) *Kiln-drying.* A weighed sample of the original wood was placed on a glass dish in a layer 1½ in. deep and the following kiln-drying schedule, which was considered suitable for 2-inch boards, was applied.

The rate of initial warming of the wood flour was 6·7° per hour at a constant humidity of 75 % up to a temperature of 73·9°. The wood was maintained at 73·9° and 75 % relative humidity for 3 hours and then the

Schedule employed in the kiln-seasoning of 80–100 mesh Silver fir.

Temperature ° C.	Relative humidity	Duration of treatment (days)	Total time (days)	Assumed moisture content %	Remarks
71·1	75	1	1	35–30	Time includes initial warming
71·1	70	1	2	30–25	—
73·9	65	1½	3½	25–20	—
73·9	60	1¾	5¼	20–15	—
76·7	55	1¾	7	15–10	—
79·4	50	1	8	10	Time includes final cooling

temperature was allowed to fall to 71·1°. Final cooling took place at the rate of 6·7° per hour.

As a result of this treatment the moisture content of the wood was reduced to 9·57 %. There was slight darkening in colour and a loss of wood substance amounting to 0·89 % of the oven-dry weight of the original wood was recorded.

(iii) *Oven-drying.* (*a*) A weighed sample of the original wood was maintained at 105° for 16 hours. The material became darker in colour.

(*b*) A similar sample of the original wood was maintained at 105° for 9 days. There was pronounced darkening in colour and a loss of wood substance amounting to 0·62 % of the oven-dry weight of the original wood was recorded.

The analysis of the original wood and the analyses of the same material after the several drying treatments are recorded in Table I. The data in Table II were obtained in order to determine the effect of 1 % NaOH on the original wood and on the wood after drying.

Table I. *Analysis of 80–100 mesh Silver fir wood before and after air-, kiln- and oven-drying.*

Material	Duration of treatment (days)	Loss due to treatment	Cold water-soluble	Hot water-soluble	Cellulose	Lignin	Methoxyl	Total pentosans	Pentosans not in cellulose	Pentosans in cellulose
Original wood	—	—	1·34	2·57	57·89	28·09	5·29	10·15	6·89	3·26
Air-dried	200	Nil	1·07	2·06	58·64	28·88	4·89	9·44	6·31	3·13
Kiln-dried	8	0·89	1·36	2·82	57·59	28·24	4·67	9·45	6·35	3·11
Oven-dried (*a*)	⅔	Not determined	1·60	2·94	57·41	28·27	4·78	9·49	6·41	3·08
(*b*)	9	0·62	2·35	4·52	57·23	28·19	4·74	9·29	6·34	2·95

Table II. *Analysis of air-, kiln- and oven-dried Silver fir wood after alkali-extraction.*

Material	Loss due to treatment	Cellulose	Lignin	Methoxyl	Total pentosans	Pentosans not in cellulose	Pentosans in cellulose
Original wood	11·14	55·98	26·52	4·01	8·59	4·97	3·62
Air-dried	12·63	55·64	26·41	3·96	7·88	4·70	3·18
Kiln-dried	10·82	55·96	26·37	4·25	8·18	4·84	3·34
Oven-dried (*a*)	12·22	54·37	26·12	3·66	7·87	4·99	2·88
(*b*)	15·07	53·38	25·30	3·93	7·27	4·37	2·90

Discussion.

Having due regard to manifest imperfections in analytical procedure, the data in Table I would, at first sight, suggest that during drying Silver fir wood suffers no radical change in composition. Critical examination reveals, however, that the data are consistent with slight though none the less definite chemical changes. For instance, in the case of air-drying, it can be seen that the yields of Cross and Bevan cellulose and of lignin are higher in dry wood than in green wood. These slight increases are accompanied by depreciations of water-soluble material and furfuraldehyde-yielding complexes respectively. Further, the loss in total pentosans represents a combined loss of pentosans in the cellulose and pentosans not in the cellulose. Since no loss in weight was observed, the depreciation in pentosans must be reflected in the increase in lignin content of the dry wood, and it must follow that the source of the accretions to the cellulose must be some material in green wood which is soluble in water. This contention derives support from the fact that as a result of air-drying, the combined losses of water-soluble material and total pentosans represent 1·49 %, and the combined additions to the cellulose and lignin amount to 1·54 % of the oven-dry weight of the original wood.

Further examination of the data in Table I shows that kiln-drying brings about a loss of wood substance which amounts to 0·89 % of the oven-dry weight of the original wood. It is significant that while no additions have been made to the cellulose, the kiln-dried wood contains approximately the same amount of water-soluble material as the original wood. The observed increase in lignin content during drying is extremely small, but it is consistent with previous work. It is interesting to note that the percentage increase in lignin content is exactly equal to the decrease in the pentosan content of the cellulose, and the suggestion thus presents itself that the drying treatment has been responsible for the decomposition of a portion of the furfuraldehyde-yielding complexes which was not associated with the cellulose in the original wood, and which under other conditions of drying might also have become associated with the lignin. This loss in furfuraldehyde-yielding material calculated as pentosans by means of Kröber's table amounts to 0·55 % of the oven-dry weight of the original wood, and thus accounts in large measure for the observed loss in weight.

For obvious reasons it has been impossible to determine directly whether the oven-drying of wood for a comparatively short period results in a loss of wood substance, but indirect evidence on this important question is afforded by the data in Table I. Oven-drying of green Silver fir wood at 105° for 16 hours results in slight, though perceptible, increases in water-soluble material and in lignin content, which are practically offset by corresponding losses in cellulose and furfuraldehyde-yielding complexes. The combined increases amount to 0·81 % and the combined losses to 0·97 % of the oven-dry weight of the original wood. The loss in weight during drying has thus been

exceedingly small. It is significant that here again the slight increase in lignin content is exactly equal to the decrease in the pentosan content of the cellulose. It would thus appear that the drying treatment has again precluded the permanent addition to the lignin of a further portion of the furfuraldehyde-yielding complexes which in the green wood was not associated with the cellulose.

The experimental data do not reveal the effect of oven-drying at 105° on wood which has already been air-dried, but if it is assumed that on oven-drying for the period commonly adopted in moisture content determinations air-dried wood would attain the same composition as green wood heated under the same conditions, it can be seen from Table I that the loss of wood substance during drying would not exceed 0·43 % of the oven-dry weight of the original wood.

When Silver fir wood is oven-dried for 9 days it is obvious from the data in Table I that incipient decomposition takes place. In the first place there is a loss of wood substance, and secondly, the dry wood contains a much higher proportion of water-soluble material than the original wood. There is a slight depreciation of cellulose and the lignin and pentosan contents are lower in the oven-dried wood than in any of the other samples.

Turning now to the data in Table II it can be seen that an interesting comparison is afforded between the alkali-solubility of the original wood and the alkali-solubility of the same material after the various drying treatments. Further, a comparison of the data in Tables I and II shows the effect of 1 % NaOH at 100° on the major wood components both before and after drying. The most striking fact illustrated by Table II is that after alkali-extraction the original, air-dried, and kiln-dried wood are approximately of the same composition, despite the fact that the alkali-solubility of the air-dried wood is greater than that of either the original or the kiln-dried wood. While alkali attacks the pentosans not in the cellulose of all the samples, the pentosan content of the cellulose exhibits a slight though perceptible increase in amount in each case. The cellulose and lignin aggregates in air-dried wood are more soluble in alkali than the corresponding aggregates in either green or kiln-dried wood. The only explanation of this fact would appear to be that the material added to these aggregates during air-drying is readily soluble in alkali. Since addition to the cellulose does not take place during kiln-drying the alkali-solubility of this component is not increased, but the alkali-solubility of the lignin is slightly increased because in this case material is added during drying. The increase in alkali-solubility of the lignin is offset by a decrease in the alkali-solubility of the pentosans not in the cellulose.

The relatively high alkali-solubility of the oven-dried wood can only be explained by the fact that incipient hydrolysis has taken place as a direct result of the high temperature treatment. This was indicated in Table I by the higher water-solubility of oven-dried wood, but the results in Table II reveal that, irrespective of the fact that after 16 hours' drying at 105° no

additions were made to the cellulose, the alkali-solubility of this component has increased. The incipient hydrolysis has apparently been confined to the carbohydrate components of the wood substance.

Prolonged oven-drying of green wood results in a pronounced increase in the alkali-solubility of the wood as a whole, which is reflected in the greater susceptibility to the reagent of the lignin as well as the carbohydrate components. In the light of previous work [Hawley and Campbell, 1927] the effect of prolonged oven-drying can only be described as a partial hydrolysis.

Conclusions.

From a comparison of the foregoing data with those obtained in the study of the drying of oak wood [Campbell and Booth, 1930] it is to be concluded that although hardwood and softwood react somewhat differently to similar drying conditions, they have one feature in common. As moisture leaves the wood, changes take place in the furfuraldehyde-yielding complexes of the wood substance. Under suitable conditions of drying the hardwood cellulose and lignin respectively are enhanced at the expense of the furfuraldehyde-yielding complexes, whereas under similar conditions only the lignin of the softwood is enhanced from this source. It is to be concluded that the material added to the cellulose of the softwood during air-drying is probably of an unstable nature, unlike true cellulose in composition, since it is recruited from water-soluble material in the green wood, and can readily be split off by dilute alkali from the air-dried wood.

In general, for both types of wood the effect of moderate heat during drying is to reduce the amount of the additions to the cellulose and lignin, while high temperatures definitely cause hydrolysis. It has become apparent from the results of research at present in progress that even the incipient hydrolysis of wood substance caused by fungi of the brown rot type has such marked effects on the physical properties of timber as the decrease of mechanical strength and a tendency to cause brittleness. Thus it becomes obvious that the woods which are chemically best suited to withstand high temperatures during drying are those which are least susceptible to hydrolysis. It has been shown in previous work that the pentosans, or rather the furfuraldehyde-yielding complexes of wood substance, are the most susceptible to hydrolysis of all the principal wood components and the suggestion has been made [Campbell and Booth, 1930] that a wood of high pentosan content would be more susceptible to heat than a wood of low pentosan content. The results of the present investigation would appear to bear this out.

In the first place, by comparison with previous data [Campbell and Booth, 1930] it can be seen that the extent of the depletion of the pentosans *per se* during drying is approximately of the same order of magnitude in the hardwood and softwood, although depletion in the hardwood takes place only in the pentosans not in the cellulose. Since, however, the softwood contains less than half the percentage of pentosans present in the hardwood, the net effect

of drying on the former is correspondingly less pronounced than it is on the latter.

Purely physical considerations render it difficult in practice to remove the greater part of the moisture from green wood without at the same time producing some of the defects commonly described in technical literature, but since rapid drying is so frequently effected by employing heat and high relative humidities, the question arises as to whether some defects may, in part, be due to chemical changes induced by unsuitable conditions of drying. For instance, it has been pointed out by Stillwell [1928] that all species of wood are not equally tolerant of heat, and the suggestion is further made that there may be a temperature for all species above which seasoning cannot proceed without damage. At the same time it is eminently feasible that there may be critical temperatures for individual species above which drying cannot proceed without causing a degree of hydrolysis which will be reflected in decreased strength of the dry wood. In this connection it should be borne in mind that apparently small changes in composition detected by current methods of analysis may cause marked changes in physical properties, and the results of the present series of investigations suggest that, since the chemical changes due to the drying of timber originate in small changes in the furfuraldehyde-yielding complexes of the wood substance, the so-called pentosans of wood substance play an important part in the determination of physical characteristics of the material as a whole.

Note on the determination of moisture by the standard oven-drying method.

The present investigation throws a new light on the question of moisture determination by the oven-drying method. It is clear that oven-dried wood is not exactly of the same composition as green wood. After drying for 16 hours at 105° the error in moisture content in the case of green Silver fir wood can amount to 0·16 %. For air-dried wood the error might be appreciably greater, and it is conceivable that the degree of error might vary from species to species. The degree of error estimated above cannot in itself be regarded as serious, but, at the same time, it would appear to be advisable in determining moisture by the oven-drying method not to prolong the drying time longer than is absolutely necessary for the samples to attain constant weight. In this way error due to incipient hydrolysis would be minimised.

The effect of 1 % NaOH at 100° on wood substance.

It was reported in a previous paper [Campbell and Booth, 1930] that after oak wood had been heated at 100° with 1 % NaOH the pentosan content of the cellulose was higher than that of the untreated wood. A similar result was later obtained with beech wood [Campbell, 1930]. This was thought to be a characteristic reaction of hardwoods as it had never been reported for a softwood. The same phenomenon has been observed, however, in the present investigation in the wood of Silver fir, although the increase in the pentosan

content of the cellulose here is by no means so great as in the hardwoods examined. These results suggest a possible difference in composition between the furfuraldehyde-yielding complexes which are associated with the cellulose of wood on the one hand and those which are not associated with the cellulose on the other. The matter will receive more detailed investigation.

SUMMARY.

1. The chemical effects of air-, kiln- and oven-drying on the wood of Silver fir have been examined in detail.

2. Air-drying is shown to result in depreciation of pentosans and water-soluble material. Lignin is enhanced at the expense of the furfuraldehyde-yielding complexes, and cellulose is enhanced at the expense of the water-soluble material of the green wood.

3. Kiln-drying precludes the additions to cellulose and part of the additions to lignin.

4. Oven-drying of green wood induces incipient hydrolysis of the wood substance.

5. An attempt has been made to confirm a previous suggestion that softwoods withstand high temperatures during drying better than hardwoods on account of their relatively low pentosan content.

6. The oven-drying method of moisture determination is subject to a slight error due to incipient hydrolysis of wood substance.

The authors wish to express their indebtedness to Mr R. A. G. Knight of the Seasoning Section, Forest Products Research Laboratory, for carrying out the kiln-drying treatment, to Prof. F. Soddy for facilities afforded in the Old Chemistry Department, Oxford University, to Sir James Irvine for criticising the manuscript, and to R. S. Pearson, Esq., Director of Forest Products Research, for permission to publish these results.

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LXXXV. A STUDY OF THE HYDROGEN ION CONCENTRATION OF THE POTATO TUBER.

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(*Received April 3rd, 1931.*)

Numerous investigations have been made on the acidity of plant sap and several methods have been employed. The hydrogen electrode has been used extensively but the time required for the system to reach equilibrium, the alteration of the biological fluid by the passage of hydrogen and the poisoning of the electrode by the plant products are obvious disadvantages of the method. Colorimetric methods are not entirely satisfactory on account of the rapid colour changes which may take place in the sap as a result of enzyme action. With the quinhydrone electrode, the precision of which has been critically examined by Morgan, Lammert and Campbell [1931], equilibrium is attained very rapidly and manipulation is simple. Plant oxidases, however, attack quinol [Chodat, 1910] and, consequently, the ratio quinol : quinone is liable to be altered. In this paper, the results obtained by rapid measurement of the acidity of tuber tissue with a special quinhydrone capillary electrode are described and compared with those obtained by other methods.

Methods.

(*a*) *Extraction of sap.* When it was desired to examine the sap, the tubers were washed in cold water and passed through a pulping machine. The mash was filtered through fine linen and the filtrate, which gave essentially the same results as the pulp, was generally employed. The pulped tubers decomposed rapidly so that the sap usually had a red-brown colour which became darker on standing.

(*b*) *Hydrogen electrode measurements.* A Hildebrand half-cell was used in conjunction with a saturated calomel electrode. About 5 cc. of the plant sap were taken for each determination and equilibrium was reached after approximately 10 minutes. The hydrogen electrode was given a fresh deposit of platinum black after each measurement.

(*c*) *Quinhydrone electrode measurements.* The quinhydrone electrode consisted of a piece of polished platinum foil which was placed in the potato sap saturated with quinhydrone. Some preliminary experiments showed that at least 0·12 g. of quinhydrone had to be added to each 10 cc. of sap in order to

obtain reproducible results. The procedure adopted was to shake the sap with sufficient quinhydrone for a few seconds, complete the potentiometer circuit with the platinum and saturated calomel electrodes and read the E.M.F. of the cell at once. The whole operation was carried out in less than 1 minute but decomposition of the phenol had already commenced. The platinum foil was washed and heated to redness in an alcohol flame after each measurement and from time to time was also cleaned in hot chromic acid solution.

A comparison of results obtained by methods (*b*) and (*c*), included in Table III, shows that in most cases the hydrogen electrode yields slightly higher p_H values. This may be due to some alteration in the sap during the passage of hydrogen or to some error in the application of the quinhydrone half-cell. The fact that the sap, if allowed to stand for about 10 minutes before the addition of the quinhydrone, gave approximately the same value as with the hydrogen electrode, suggests that the time required by the system to reach equilibrium is the important factor.

(*d*) *Micro-electrode measurements.* Reproducible results were readily obtained with the quinhydrone electrode, but the use of pulp in any method removes the possibility of studying the variation in acidity throughout the tuber. To meet this difficulty a special electrode was constructed [Robertson and Smith, 1930], which enabled measurements to be made at any desired point on a section of the plant tissue. A few results obtained by means of this electrode and by the method described under (*c*) are given in Table IV. The p_H of the sap was always greater than that observed with the micro-electrode for the tissue, which was probably due to loss of carbon dioxide in the preparation of the pulp. Ingold [1929] observed a reduction of 0·3 p_H unit when the tuber sap was brought into equilibrium with concentrations of carbon dioxide such as have been recorded for the intercellular spaces of the tuber. In addition, the oxidase activity of the disintegrated tissue is very great and the formation of melanin and its intermediate compounds from tyrosine [Raper, 1928] may also reduce the acidity.

Variation in acidity throughout the tuber.

Although some data are available on the hydrogen ion concentration gradients of the sap of plants [Gustafson, 1924], no such observations seem to have been made on the potato tuber. Attention was directed to a study of the changes which take place during the formation, storage and sprouting of the tuber.

(*a*) *During growth period.* A number of healthy and typical plants was selected and examined at intervals throughout the summer. The tubers were cut along a plane through an eye and the heel. Readings were taken with the micro-electrode at points in the middle and 1–2 mm. below the eye and heel. The results for two varieties given in Table I are typical of a large number of observations and each figure is an average value for four tubers.

Table I. *Variation of tuber* p_H *with stage of growth.*

Variety	Date of sampling ...	30. vi. 30	21. vii. 30	16. viii. 30	30. ix. 30
Duke of York	"Rose" end	6·19	6·04	5·90	5·65
	Centre	6·20	6·08	5·75	5·71
	"Heel" end	5·53	5·90	5·85	5·73
Epicure	"Rose" end	6·08	6·13	5·81	5·68
	Centre	6·06	6·09	5·88	5·75
	"Heel" end	5·78	5·82	5·80	5·80

The first readings were made with tubers weighing 5–15 g. and it was observed that the underground stem attaching the tuber to the parent plant had approximately the same p_H as the heel of the tuber. The final sampling was made after the plants were completely ripe, the haulms having withered away some time previously. The results show that all parts of the tuber, except the heel, become more acid as the plant ripens. The p_H of the heel rises quickly and then falls off gradually. There is a p_H gradient across the young tuber, the heel being more acid than the rose end. In the mature tuber the reverse holds good. This change coincides with the physiological development of the tuber, for in the early stages of growth the active part is at the heel where nutrients are received from the plant, whereas in the mature state this process has ceased and the eyes develop rapidly.

(*b*) *During rest period.* A large number of tubers was examined between the time of harvesting and the appearance of sprouts. Observations were made at eleven points on a section of each tuber, both cortex and medulla being represented. In the majority of cases the rose end was slightly more acid than the heel end, the centre being intermediate. The values given in Table I for the last sampling are typical in this respect. In many tubers, however, no difference was noted between the rose and heel ends, while in a few isolated cases the heel was slightly more acid than the rose end. Generally, the average value for all the points approximated closely to that for the centre of the tuber.

(*c*) *During sprouting period.* In March a number of dormant tubers was exposed to daylight and allowed to sprout. At certain intervals a few tubers were cut down through a sprout and eye to the heel and the acidity was determined at a number of positions across the section. Tubers of nine varieties were examined and all yielded similar results. In Table II the average values for three tubers of the variety Duke of York are given.

Table II. *Variation of* p_H *during sprout formation.*

Position on tuber or sprout	20. iii. 30	16. iv. 30	15. v. 30	24. vi. 30
Growing tip of sprout	4·28	4·30	4·38	4·51
Middle of sprout	4·45	4·55	4·62	4·90
Tuber end of sprout	4·25	4·36	4·54	5·09
Surface of "eye"	4·58	4·73	5·70	5·85
2 mm. below surface of "eye"	5·31	5·60	5·86	—
5 mm. below surface of "eye"	5·61	5·67	5·83	—
10 mm. below surface of "eye"	5·75	5·74	5·86	—
Middle of tuber	5·74	5·71	5·78	5·80
"Heel" end of tuber	5·80	5·85	5·86	5·88

The following is a description of the sprouts:

20. iii. 30.—Sprouts soft and light green, and about 0·5 cm. long.
16. iv. 30.—Sprouts hard and dark green, 0·5–1·5 cm. long.
15. v. 30. —Sprouts thicker, 2·0–2·5 cm. long, small leaves forming.
24. vi. 30.—Sprouts very thick and hard, 3 cm. long, small leaves and shoots formed. Tuber soft.

When sprouts appear, the region round the active eyes is very acid, but gradually becomes less acid as the sprouting progresses until June, when the p_H is practically uniform throughout the tuber. The growing tip of the sprout retains its relatively high acidity throughout the growth of the plant; in a young field plant it had p_H 4·46 while in an older plant the p_H was 4·65.

These variations in p_H through the tuber are to be expected if consideration is given to the different functions of the various parts. The actively growing parts are usually more acid than the fully developed members. On the other hand, the acidity of the tuber increases with maturity, which may be accounted for by the fact that, according to Appleman and Miller [1926], the protein in the young tuber gives place to non-protein- and amino-nitrogen in the mature tuber.

Factors influencing the p_H *value of the mature tuber.*

(*a*) *Environment.* Tubers of six common varieties, grown at seven places in Midlothian, were examined immediately after harvesting. The altitudes of the different places varied from 100 to 600 feet above sea level; the different soils varied in texture from sandy loams to clay loams and in p_H from 4·9 to 7·3. There was no obvious relationship, however, between environment and the acidity of the tuber. The maximum variations of individual values for all sources were as follows: Duke of York, 5·70–5·89; Epicure, 5·70–5·85; Great Scot, 5·65–6·11; Golden Wonder, 5·70–5·85; Majestic, 5·50–5·73; Ally, 5·67–5·84.

In order to investigate more fully the effect of soil reaction upon the acidity of the tuber, a crop of variety King George, grown on a series of specially treated plots on a soil of p_H 4·0, was examined. The p_H of the tubers was measured by methods (*b*) and (*c*) and the results are summarised in Table III.

Table III. *Relation between* p_H *of soil and tuber sap.*

		p_H of tuber sap	
p_H of soil	No. of plots	Method (*b*)	Method (*c*)
4·0–4·5	4	5·69	5·66
4·5–5·0	11	5·68	5·64
5·0–5·5	11	5·66	5·63
5·5–6·0	5	5·69	5·63
6·0–6·5	3	5·68	5·63
6·5–7·0	2	5·62	5·68

In 1929, five plots were laid down on a soil having p_H 5·5. Two plots were made more acid by treatment with flowers of sulphur and two more alkaline by treatment with calcium hydroxide. Two varieties were grown on each plot.

In autumn, samples of tubers were lifted, washed and dried and then examined as follows. Ten tubers were selected at random from each sample and cut through the rose and heel ends. The acidity was determined at various points on the section by means of the micro-electrode. The small injuries made by the electrode were then cut out, the tubers were pulped and the p_H of the sap was determined by method (*c*). The average results are given in Table IV, the p_H values of the soil being those obtained during September.

Table IV. *Relation between* p_H *of soil and tuber.*

		Average p_H of tubers			
		Duke of York		Great Scot	
Plot	p_H of soil	(*d*)	(*c*)	(*d*)	(*c*)
A	8·1	5·70	5·77	5·68	5·73
B	7·5	5·68	5·75	5·69	5·73
C	5·5	5·70	5·74	5·69	5·70
D	5·1	5·70	5·73	5·68	5·74
E	5·0	5·71	5·77	5·68	5·72

It is obvious from the above results that the p_H of the tuber is not influenced by that of the soil. Hoagland and Davis [1925] have obtained similar results for other plants grown in culture solutions, but other workers, for example Haas [1920], have found a certain relationship between the acidities of soil and plant sap.

(*b*) *Variety.* In addition to the p_H figures already given for different varieties, the following were obtained: Arran Consul, 5·64; Field Marshal, 5·74; Kerr's Pink, 5·75; King Edward, 5·95. The differences between varieties were thus not significantly greater than the variations found among tubers of the same variety.

(*c*) *Disease.* A number of tubers affected by various diseases was collected and compared with normal tubers of the same variety. The influence of virus infection was also studied. The results are presented in Table V.

Table V. *The effect of disease upon the* p_H *of tubers.*

			Affected tuber	
Variety	Nature of infection	Normal tuber	Healthy part	Diseased part
Great Scot	Corky scab: *Spongospora subterranea* Lagerh.	5·70	5·65	4·35
Duke of York	Common scab: *Actinomyces scabies* (Thaxt.) Güssow	5·75	5·70	4·58
Epicure	Sprain: *Bacterium rubefaciens*	5·73	—	5·60
Epicure	Blackleg: *Bacillus atrosepticus* van Hall	5·73	5·68	5·65
Duke of York	Blight (fresh): *Phytophthora infestans* (Mont.) De By.	5·75	5·75	5·83
Duke of York	Blight (old)	5·75	5·70	5·38
Duke of York	Wart disease: *Synchytrium endobioticum* (Schilb.) Perceval	5·75	5·65	5·02
Ally	Mosaic	5·80	5·70	
	Crinkle	5·80	5·60	
	Leaf Roll	5·80	5·85	
Arran Comrade	Mosaic	5·64	5·47	
	Leaf Roll	5·64	5·70	

In diseased tubers a very acid region was observed around the affected part. In mild forms the disease did not seem to affect the rest of the tuber but in severe cases a slight increase in acidity was noted throughout the tuber. The slight decrease in acidity due to Leaf Roll and the large increase due to Wart Disease confirm results reported by Boas [1919] and Weiss and Harvey [1921] respectively. Severe forms of mosaic, such as crinkle, gave rise to an increase in acidity.

(*d*) *Storage.* A number of tubers of each of six varieties was stored in three different ways, *viz.*: (1) in a pit in the open, (2) in a cool, well-ventilated storehouse, (3) in a refrigerator maintained at 2°. After a few months the average p_H values were as follows:

Original value (October)	5·76
After storage (2)	5·84
,, (1) immediately	5·49
,, (1) after 24 hours at room temperature	5·88
,, (3) immediately	5·39
,, (3) after 24 hours at room temperature	5·83

The tubers from the pit and refrigerator were more acid than before storage, but, on being allowed to stand at room temperature for a day, acquired a p_H slightly higher than the original. This was probably due to increased respiratory activity of the tissue immediately after removal from such storage conditions.

Summary.

The hydrogen and quinhydrone electrodes have been used for p_H measurements of the pulp of the potato (*Solanum tuberosum*), and a micro-electrode enabled measurements to be made at different points of the tissue.

The acidity is not uniform throughout the tuber but depends upon the function of the different parts at different periods in the life of the plant. In the early stages of development the underground stem and the heel end of the tuber are most acid and the average acidity of the tuber increases with maturity. In the dormant state the acidity does not vary much in different parts of the tuber but when sprouting begins the active eyes are most acid.

The acidity of the tuber is independent of environment and is not influenced by large variations in soil acidity.

The differences in acidity due to variety and storage are not significant.

Comparatively large changes in acidity are associated with certain diseases.

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LXXXVI. A NOTE ON THE INHIBITORY EFFECT OF MONOIODOACETIC ACID ON LACTIC ACID PRODUCTION BY CANCER TISSUE.

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Lundsgaard, in recent work [1930, 1] has shown that monoiodoacetic acid prevents the production of lactic acid in contracting muscle. Later [1930, 2] he found that, with concentrations of iodoacetic acid which completely inhibit glycolysis, the oxidising systems of yeast and muscle remain unimpaired. Warburg has shown that tumour cells derive a large part of their energy for growth from glycolysis. If this glycolysis of tumour could be inhibited by iodoacetic acid as is that of muscle, the growth of the tumour might be checked. Lundsgaard states that he is testing the effect of iodoacetic acid on the growth of tumours in mice by injecting it into the living animal. In order to see whether such inhibition of growth might be expected in tumours, we tried the effect of iodoacetic acid on glycolysis of tumours *in vitro*. So far as is known, the mechanism of glycolysis in tumours differs considerably from that in muscle, the tumour glycolysis appearing to be independent of hexosephosphate [Harrison and Mellanby, 1930]. Lundsgaard showed that in muscle under the influence of iodoacetic acid hexosephosphate is synthesised but not broken down. Since the production of lactic acid in the tumour cell appears to be independent of the breakdown of hexosephosphate, it seemed to us possible that iodoacetic acid might not produce the same inhibiting effect upon the lactic acid production by tumours as it produced upon that by muscle. The experiments given in the table show, however, that iodoacetic acid causes a large inhibition in the production of lactic acid by tumours *in vitro*. As yet we are unable to explain by what mechanism the iodoacetic acid causes such inhibition in tumour glycolysis.

Experiments *in vitro*.

Mouse carcinoma 63 was cut into thin slices, weighed and dropped into flasks containing 50 cc. bicarbonate Ringer's solution (brought to p_H 7·8 by passing through it a mixture of 5 % CO_2 and air), 5 cc. 9·6 % glucose (final concentration = 0·8 %), and 5 cc. either of water or of a neutralised solution of iodoacetic acid dissolved in 0·8 % NaCl. 30 cc. were then removed into a solution of trichloroacetic acid for the determination of the amount of lactic

acid already present[1]. The flasks were then stoppered and shaken for 3 hours at 37° and, after removal of proteins and carbohydrates, the lactic acid was estimated by the method of Friedemann, Cotonio and Shaffer [1927].

Table I. *Aerobic glycolysis of tumour tissue as affected by iodoacetic acid.*

		mg. lactic acid per g. dry weight tissue mg.
Exp. 1.	Control	39·1
	Iodoacetic acid 2 mg. in 30 cc.	14·5
Exp. 2.	Control	41·4
	Iodoacetic acid 4 mg. in 30 cc.	7·3
Exp. 3.	Control	34·6
	Iodoacetic acid 4 mg. in 30 cc.	1·0
Exp. 4.	Control	30·3
	Iodoacetic acid 1 mg. in 30 cc.	8·9
	,, 2 mg. in 30 cc.	5·6
	,, 4 mg. in 30 cc.	3·0

In Exp. 3, the tissue had been shaken for an hour in bicarbonate Ringer's solution and then put into fresh Ringer's solution with glucose and shaken with and without iodoacetic acid.

Experiments *in vivo*.

Several experiments were done in which iodoacetic acid was injected in a sublethal dose into mice bearing tumour 63. After a given time, the animals were killed and the glycolysis of the tumour compared with that of tumours from uninjected animals. In order to make the control as valid as possible, both the injected and uninjected animals bore tumours from the same inoculation. Since there is some variation in the glycolytic values of untreated cancerous tissues from different animals even when the tumours are produced from the same parent tumour, it is evident that only very large differences between the degree of glycolysis of the tumours of the injected and the uninjected animals would be significant. Glycolytic values from a number of tumours were compared to give the control figure. The glycolytic value of 5 tumours of the same generation ranged between 31 and 43 mg. lactic acid per g. of tissue, and an average value of 37 mg. was taken for the control figure. The animals were given a subcutaneous injection (on the side opposite the tumour) of 0·2 cc. of 1 % iodoacetic acid (previously neutralised) and were killed from 1 to 2 hours afterwards. Their tumours were removed, sliced and put into sugar-containing bicarbonate Ringer's solution, and the experiment was run as already described. Animals A and B, killed respectively 1 and 2 hours after injection of iodoacetic acid, appeared to be normal and showed no symptoms of iodoacetic acid poisoning. Animal C, on the other hand, was almost moribund when killed 2 hours after injection.

[1] In the figures given in the tables this amount of preformed lactic acid has already been subtracted from the values both for the control solutions and those containing iodoacetic acid.

Table II. *Aerobic glycolysis of tumour slices after injection of iodoacetic acid.*

	Lactic acid per g. dry weight tissue mg.
Animal A	48·8
Animal B	56·5
Animal C	12·9
Control	37·0

The figures for the tumour glycolysis of the injected animals are an average of three experiments done on each tumour.

These results, although varying considerably from the control, at least indicate that when the dose of iodoacetic acid is sublethal, there is no inhibitory effect upon lactic acid production of the tumour after a single injection, but when the dose causes acute symptoms of poisoning, the lactic acid production of the tumour is strongly inhibited.

SUMMARY.

1. The aerobic production of lactic acid by tumour slices is inhibited by iodoacetic acid.

2. A few experiments, on mice, in which a single injection of iodoacetic acid was given, show that no inhibition of lactic acid production in tumour occurs provided that the dose is sublethal.

We are indebted to the Yorkshire Cancer Research Fund for the expenses incurred in carrying out this work.

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LXXXVII. THE BLACKENING OF POTATOES AFTER COOKING.

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(*Received March 18th, 1931.*)

It is well known that potatoes after cooking sometimes darken considerably on the surface. This blackening is distinct from the darkening which occurs with raw potato on exposure to air. In the latter case, there is no doubt that the darkening is due to enzymic oxidation, the oxidisable substance being a phenolic substance of the nature of catechol or other *o*-dihydroxybenzene derivative [Onslow, 1919, 1920] or tyrosine [Bertrand, 1896; Gallagher, 1923].

The darkening of potatoes after cooking appears to be a matter of some considerable importance to those concerned in the growing and sale of potatoes. At the request of the Ministry of Agriculture some experiments were carried out in the Household Arts Department of this College with reference to methods of cooking potatoes in order to find out whether such darkening could be avoided. Some of the potatoes supplied by the Ministry of Agriculture were obtained by the author in order that the problem might be investigated from a chemical standpoint.

In the first experiments "King Edward" and "Great Scot" potatoes were employed and later "Majestic" potatoes were used. Several batches of potatoes of these three varieties have been used in the later experiments, but in the experiments to be described in this paper the potatoes which do not blacken on cooking will be referred to as potatoes A, and those which blacken as potatoes B.

One of the first objects of the present investigation was to devise, if possible, a simple test by means of which it would be possible to tell by examination of a raw potato whether or not a similar potato would blacken on cooking. It is believed that such a test has been discovered. It is described below under the action of nitrous acid on potatoes.

In preliminary experiments, tests were made with various reagents to ascertain if any great difference could be detected between those potatoes which blacken after cooking and those which do not.

Enzymic oxidation.

Transverse sections about 5 mm. thick of potatoes A and B were peeled thinly and exposed to air for one day (in some cases two days) in a bell-jar

containing some chloroform and some water. In both cases very dark brown colours were produced on the surfaces exposed as shown in Plate III, Figs. 1 and 2. Thus enzymic oxidation shows no difference between the two varieties.

The action of nitrous acid.

It was found that all potatoes examined contain a substance (or substances) in greatly varying amounts, which on treatment with nitrous acid followed by an alkali, gives a fine red colour. The amount of the red substance produced in this test was found to vary exactly with the amount of blackening which takes place on cooking. The test is carried out as follows. A transverse section of potato about 5 mm. thick is peeled thinly and covered with 7 % sodium nitrite solution (about 25 cc.) in a small porcelain basin. About 2 cc. of dilute hydrochloric acid (1 volume of concentrated hydrochloric acid to 2 volumes of water) are added and the mixture left for 5 minutes. The liquid is then poured off and the section of potato covered with 16 % sodium hydroxide solution (about 25 cc.). The red colour develops in about 5 minutes, at first chiefly on the outer and inner edges of the fibro-vascular layer of the potato, then through the whole of this layer, but it often extends towards the centre. It is strongly marked where there are eyes in any potato. After some time the coloured substance is partly extracted by the sodium hydroxide solution.

This reaction has not yet been investigated fully. It may be that the nitrous acid reacts with a primary amino-compound and that coupling of the diazo-compound thus produced takes place with a phenoxide on the addition of the sodium hydroxide. The colour may, however, be due partly or entirely to reactions between the nitrous acid and lignocellulose.

The red colour of the sodium hydroxide solution of the substance produced in the reaction is changed on addition of dilute hydrochloric acid and restored on making alkaline. With concentrated hydrochloric acid, on heating, the red substance is decomposed, as the colour is not restored on again making the solution alkaline.

It appears very probable that the production of this colour in the test described above is in some way connected with the blackening which takes place after cooking. If for example the fibro-vascular layer of a potato B, which gives most colour in the test, is completely removed before cooking very little blackening of the remainder is usually noted after cooking.

Photographs of sections of potato after applying the nitrous acid test are shown in Plate III, Fig. 3, for potato A which does not blacken after cooking, and in Plate III, Fig. 4, for potato B, which blackens.

In Plate III, Figs. 6, 7 and 8, photographs are shown of pieces from the surface of the same end of one potato B. Fig. 6 shows a piece of the potato after cooking by steaming, Fig. 7 raw, untreated potato, and Fig. 8 raw, treated with nitrous acid and sodium hydroxide. A resemblance between the photographs Figs. 6 and 8 will be noted, but of course the portion of potato represented in Fig. 6 is black and in Fig. 8 red.

Fig.1.

Fig.2.

Fig.3.

Fig.4.

Fig.5.

Fig.6.

Fig.7.

Fig.8.

If the water in which potatoes are boiled is treated with nitrous acid and sodium hydroxide, coloured solutions are obtained—deep colours with water in which potatoes which blacken have been boiled and faint colours if the potatoes do not blacken.

The blackening after cooking.

It is extremely probable that this blackening is due to oxidation, but it cannot be due to enzymic oxidation, as during cooking the temperature of a potato is at about 100° for about 20 minutes. Whether the blackening after cooking is due to the oxidation of a phenol or amine has not yet been ascertained. Experiments in support of these statements as to blackening after cooking being due to oxidation are as follows.

(*a*) If a portion of potato B, which blackens after cooking, is placed immediately it is cooked in a gas free from oxygen and thus allowed to cool in absence of air it does not blacken.

(*b*) If a piece of cooked potato is placed in a bell-jar containing chloroform and water no effect such as that described above under enzymic oxidation takes place.

The effect of traces of iron on the colour of cooked potato.

It was noticed in the course of this work involving the cooking of potatoes in various ways that a black stain was occasionally obtained on a cut surface of potato when such a stain was not expected.

In one experiment some potatoes were being cooked by steaming on filter-paper in a steamer made from tinned sheet iron. The perforations in the steamer had apparently been made after the sheet iron had been tinned and the edges of the holes were rusting. It was noticed that black marks were obtained on the potatoes (after cooling in the air for some time) which coincided exactly in position with the holes in the steamer. A section of potato B showing these marks is shown in Plate III, Fig. 5.

The explanation of the black marks is probably as follows. The iron compounds from the sides of the steamer holes evidently pass through the filter-paper (as shown by rust marks on standing) and coming in contact with the potato act either catalytically in promoting oxidation, or else combine with the phenolic substances present to produce coloured substances. Most probably both actions take place. Evidence of combination of iron compounds with the phenols is obtained from the fact that somewhat violet colorations are sometimes obtained in such experiments. Ferric oxide alone does not appear to promote blackening.

It is known that it is a ferrous compound that is involved in the catalytic activity of iron in biological oxidations, and since it is iron in the process of rusting (when a ferrous compound will be present) which causes most blackening it appears that the effect of iron in promoting blackening is in part catalytic.

The effect of iron in the process of rusting on the production of blackening after cooking is well shown by steaming a peeled potato in which are inserted some newly cleaned iron nails and allowing the potato to remain exposed to the air some hours after cooking. In view of the extensive use of iron in cooking vessels the action of this metal in causing blackening after cooking must not be overlooked.

The effects of ammonia in steaming potatoes and of acid in the cooking water.

Owing to the well-known effect of ammonia in promoting atmospheric oxidation of polyhydric phenols it was found, as was expected, that potatoes steamed over water containing a little ammonium carbonate were dark in colour after cooking. The effect of the hydrogen ion concentration of the cooking water on the colour of cooked potato is to be investigated, but it has already been found that if potatoes B are cooked in water containing a little acetic acid they are usually a better colour than if cooked in tap-water.

Similarly a section of potato after soaking in dilute acid gives less colour in the nitrous acid test than a piece of the same potato which has been soaked in water only. This might point to a basic substance being concerned in the blackening process. If the acid solution and water used for soaking are subjected to the nitrous acid test a strong reaction is obtained in the acid solution, but not with the water.

Further work on this problem is in progress.

SUMMARY.

A simple test is described by means of which it is considered possible to tell whether or not potatoes will blacken after cooking.

The cause of this blackening as distinct from darkening due to enzymic oxidation is discussed, and attention is drawn to the fact that iron may have a pronounced influence on the degree of blackening observed.

My thanks are due to Miss J. Lindsay and Miss H. Tress of the Household Arts Department of this College for samples of the potatoes used in these investigations.

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LXXXVIII. CYCLIC CHANGES IN THE GLYCOGEN CONTENT OF THE LIVER AND THE MUSCLES OF RATS AND MICE.

THEIR BEARING UPON THE SENSITIVITY OF THE ANIMALS TO INSULIN, AND THEIR INFLUENCE ON THE URINARY OUTPUT OF NITROGEN.

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(*Received April 9th, 1931.*)

The physiological changes in the glycogen content of the liver have generally been considered to be the result of changes in the carbohydrate supply of the body. It was formerly regarded as established that glycogen accumulates in the liver after ingestion of food and is eliminated from the liver by fasting, especially if combined with muscular exercise. Accordingly fasting and muscular exercise have been the principal means adopted for removing the glycogen from the liver as well as from the muscular tissue. At a very early date, however, observations were made indicating that the mechanism of this function of the liver was more complex than had been supposed.

Thus it is known that glycogen reappears in the liver and the muscles of rabbits completely deprived of their glycogen by means of strychnine convulsions. If the animals are kept anaesthetised glycogen reappears in the liver within 20 to 28 hours. Pflüger [1903] suggested the possibility of a migration of glycogen from the bony and cartilaginous parts of the body. In his excellent paper on this topic Rolly [1905] indicated the possibility of glycogen formation from body proteins. On the third to fifth day of fasting, rabbits completely deprived of their glycogen by means of strychnine convulsions showed not only the reappearance of glycogen in the liver and the muscles, but also an increase in the nitrogen output through the urine. In Rolly's experiments the excess nitrogen excretion, indicating an increased protein metabolism, occurred at a time when there was no reason to suspect a pre-agonal destruction of proteins.

These early investigations tend to show that the accumulation of glycogen and its disappearance from the liver cannot be explained solely by the storage and mobilisation of the available carbohydrate material. As is contended also by Macleod in his monograph on carbohydrate metabolism, other factors,

hitherto not well-known are involved in this mechanism. Such a view is furthermore supported by recent experimental findings.

In recent times a most valuable contribution to our knowledge of glycogen metabolism has been made by Forsgren [1928]. He has observed cyclic changes in the glycogen content of the liver of rabbits, the maximum being reached at night and the minimum during the daytime. These very striking changes are to a great extent independent of nutritional conditions. Early in the day glycogen may disappear from the liver in spite of an abundant food supply, whilst at night there is an accumulation of glycogen, even if the animals are fasting. There is a corresponding periodicity in the production of bile, the latter reaching its maximum during the day, when the glycogen content of the liver is low. Forsgren obtained these results by histochemical investigations, including staining of the bile-acids and the glycogen in the liver of animals killed at different times of the day. Forsgren's results have been confirmed by chemical analysis of rabbit's liver made by two of the authors of this paper.

In order to check Forsgren's results, we conducted experiments on a rather large scale, analysing the glycogen content of the livers of a considerable number of mice and rats at different times of the day. What particularly aroused our interest in this problem was the observation that mice used in the assay of insulin consistently show a greater resistance to insulin in the evening than at noon. We thought that the diminished sensitivity of the animals to insulin in the evening might be due to an accumulation of glycogen in the liver. This possibility has been suggested by Forsgren himself [1929].

This periodicity, if established, would moreover have an important bearing on our views of carbohydrate metabolism. It is now generally assumed that in a fasting animal the glycogen content of the liver remains constant at a low level or slowly decreases. Thus Cori and Cori [1928], in their studies of the glycogen metabolism, contend that during the second day of fasting (*i.e.* between the 24th and 48th hours) a very minute amount of glycogen only can have been removed from the liver of the rat and converted into sugar. The figures found for the glycogen content of the liver were almost identical on the two different days. This constancy, however, would be quite illusory, had there been in the meantime a considerable increase of the liver glycogen followed by a decrease. The conclusion drawn in regard to the sugar metabolism during that period would consequently be erroneous. The same remark applies to many investigations of recent date on the action of insulin and adrenaline, in which the glycogen content of the body has been studied for periods ranging from 24 to 48 hours, without knowledge of these cyclic changes.

By our experiments on mice and rats we confirmed Forsgren's findings so far as concerns this periodicity in the accumulation of glycogen in the liver. We also found that it is largely independent of nutritional conditions. In spite of 40 hours' fasting a large number of the rats showed an increase in the liver-glycogen during the night. None of the rats, which had fasted more than

24 hours, if killed during the daytime, had any appreciable quantity of glycogen in the liver. The results are thus fully in agreement with the findings of Forsgren in his experiments on rabbits.

In the experiments with insulin the increase in the resistance of the mice to the hormone was found to coincide with the accumulation of glycogen in the liver. In the evening a smaller number of animals showed convulsions than at noon, and at night the dosage of insulin had to be doubled in order to induce severe intoxications in the same percentage of animals.

Having, by our first series of experiments, established the periodicity in the accumulation of glycogen in the liver, we proceeded in the subsequent series to examine also the glycogen content of the muscles. The close connection between the glycogen content of the liver and that of the muscles has been well established, especially by the studies of Cori and Cori [1929]. These authors emphatically assert that glycogen passes from the muscles to the liver and *vice versa*, the lactic acid of the blood and the blood-sugar respectively being the intermediate compounds. On this basis it seemed reasonable to presume that the glycogen content of the muscles would show a periodicity similar to that of the liver. In fact, in our three series of experiments, in which the entire body of the animals (mice) was examined for glycogen in accordance with the method of Lesser, the glycogen content of the muscles was found to be higher during the night than during the day. The difference, however, is not so marked as in the case of the liver. This seems to indicate that the glycogen formed during the night originates from the liver.

In regard to the material which gives rise to this periodical accumulation of glycogen, it may be taken for granted that an important part is played by the carbohydrates. How far the proteins also participate in this synthesis of glycogen is a problem of considerable interest. As stated above, the view that the proteins do contribute to the formation of glycogen has been emphasised by various investigators. The fact that the periodical formation of glycogen is but slowly affected by moderate fasting likewise indicates that the body proteins may participate in the process. If this is actually the case, the urinary output of nitrogen should be affected by the process. For an increased deamination of amino-acids, in connection with the accumulation of glycogen, would naturally result in an increased nitrogen output. As, so far as we know, cyclic changes in the urinary nitrogen output of experimental animals have not previously been demonstrated, we thought it desirable to investigate this question by experiments on rabbits. The results make it evident that in rabbits the nitrogen output does show a periodical variation parallel with that of the liver-glycogen.

Experimental.

The glycogen content of the liver was determined in seven series of experiments on mice and one series of experiments on rats. In each series 120 or 90 animals were taken. Either 10 animals were killed every 2 hours or 15

every 4 hours, beginning at 8 o'clock in the morning. All the animals belonging to one series were kept in a large cage under uniform conditions.

In two of the mouse series the animals were abundantly supplied with food and water. The stomachs of the mice on dissection were always filled with food. In four of the series each animal had fasted 10 hours before being killed. Previously to the latter experiments 10 animals had been placed every 2 hours in smaller cages, supplied with water only. In one of the mouse series the food had been removed 10 hours before the beginning of the experiment, so that those last killed had fasted 34 hours. The rats had fasted 24 hours before the beginning of the experiment.

The animals after weighing were killed by decapitation. The liver was rapidly removed, weighed, cut in pieces and placed in a centrifuge tube together with 1 cc. of 60 % potassium hydroxide. The tube was immersed in a boiling water-bath. The liver of a mouse is sufficiently large for a single analysis. Of the rat's liver about 1 g. was taken.

Where the glycogen content of the whole animal was determined in accordance with the method of Lesser, the mouse, after the removal of the liver, was immediately cut up with a pair of scissors, and placed in a large centrifuge tube, containing 20 cc. of hot potassium hydroxide solution. The alkaline solution, on heating in a water-bath, quickly penetrated the material. The heating was continued for 3 hours; residual bony material was removed before the precipitation with alcohol.

After the hydrolysis 1 cc. of the potassium hydroxide solution was treated with 2 cc. of water and 8 cc. of 95 % alcohol. The precipitate was washed in the centrifuge tube 3 times with 66 % alcohol, 3 times with 95 % and once with absolute alcohol. After washing with ether and drying, it was hydrolysed by heating for 3 hours in a water-bath with 5 % hydrochloric acid. The sugar determination was made according to the method of Bertrand.

By the method used 2–5 mg. glycogen were recovered with a loss not exceeding 3 %. In comparing these results with the figures found on analysis of 25 g. of the liver according to the macro-method, the differences between the figures were found to be less than 5 %.

In the series where insulin was injected into mice, the animals were taken from a stock of several hundreds kept in a large box. In two of the series (Figs. 3 and 4) 50 animals were taken out of the box 10 hours before they were injected with insulin. They were supplied with water only. In one series (Fig. 6) all of them had been deprived of food at 10 p.m. on the preceding day. In each of the experiments recorded in the figures 50 animals were injected with an amount of insulin, known to cause convulsions in 50 % of cases within 2 hours. The amount of insulin necessary was 0·0062–0·0075 units per 20 g. body weight, which was dissolved in 0·5 cc. of saline and injected subcutaneously. The temperature was kept constant at 38°.

The experiments concerning the urinary nitrogen output were performed on female rabbits. The animals were kept in small cages which did not permit

free movements. The cages were provided with bottoms of strong wire netting and were placed in aluminium bowls for the collection of urine. The urine was collected twice daily at 8.30 a.m. and 8.30 p.m. On each occasion the bladder was rinsed with water through a metal catheter. In order that the animals might be supplied with water 60 to 80 cc. of saline was at the same time injected subcutaneously. The urinary excretion was thus followed during a period of 6 days, throughout which the animals were kept fasting. The temperature and the body weight were recorded every other day.

Results and conclusions.

Fig. 1 shows the glycogen content of the liver of animals (mice) abundantly supplied with food. The glycogen content was much higher during the night

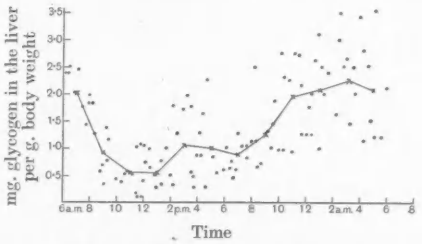

Fig. 1. Glycogen content of the liver of mice abundantly supplied with food.

than during the day. The average figures for 10 animals were about 100 % higher in the night than in the day. In Fig. 1 (Table I, A) the figures for the liver-glycogen of the 50 animals killed between 10 a.m. and 8 p.m. were

Table I. *Cyclic variations in glycogen of mice and rats under different conditions.*

(Figures represent mg. glycogen/g. body weight (and average deviation from mean) determined in groups of 10 animals.)

Time ...	8 a.m.	10 a.m.	12 noon	2 p.m.	4 p.m.	6 p.m.
A. Mouse-liver; animals well-fed	0·93 (±0·177)	0·56 (±0·139)	0·62 (±0·077)	1·10 (±0·213)	1·02 (±0·207)	0·86 (±0·093)
B. Rat-liver; animals fasted 24 hrs. before and during experiment	0·028 (±0·00225)	0·034 (±0·00438)	0·033 (±0·0051)	0·031 (±0·0062)	0·027 (±0·0033)	0·115 (±0·0348)
C. Mouse-liver; animals fasted 10 hrs.	2·22 (±0·203)	1·67 (±0·248)	1·20 (±0·244)	0·54 (±0·161)	—	0·38 (±0·049)
D. Mice as under C. Body exclusive of liver	0·57 (±0·082)	—	—	0·49 (±0·078)	—	—

Time ...	8 p.m.	10 p.m.	12 midnight	2 a.m.	4 a.m.	6 a.m.
A. Mouse-liver; animals well-fed	1·28 (±0·184)	1·94 (±0·243)	2·06 (±0·216)	2·51 (±0·243)	2·04 (±0·264)	2·08 (±0·125)
B. Rat-liver; animals fasted 24 hrs. before and during experiment	0·036 (±0·0047)	0·061 (±0·0195)	0·076 (±0·0261)	0·139 (±0·0272)	0·104 (±0·0329)	0·083 (±0·0203)
C. Mouse-liver; animals fasted 10 hrs.	0·34 (±0·062)	—	1·03 (±0·158)	—	0·81 (±0·094)	1·57 (±0·099)
D. Mice as under C. Body exclusive of liver	0·25 (±0·0)	—	0·47 (±0·033)	—	0·62 (±0·0026)	—

0·83 ± 0·0679 mg./g. body weight and for the 59 killed between 8 p.m. and 8 a.m. 1·97 ± 0·0986. Thus under normal conditions glycogen accumulates in the liver of the mouse during the night.

Similar changes occur in the liver even if the animals are deprived of food. They could be demonstrated in mice after 10 hours' fasting and in rats which had been kept without food for 40 hours. Fig. 2 shows the results

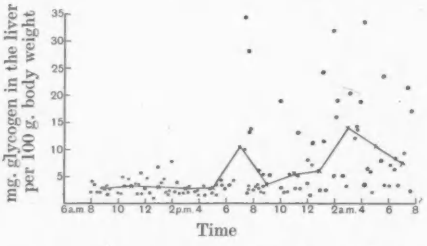

Fig. 2. Glycogen content of the liver of rats, which had fasted 24 hours before the experiment and were kept fasting.

obtained with rats which had fasted 24 to 48 hours. In the daytime the liver contained about 3 mg. glycogen per 100 g. body weight, whereas the figures during the night were 8–10 mg. The 70 animals killed between 10 a.m. and

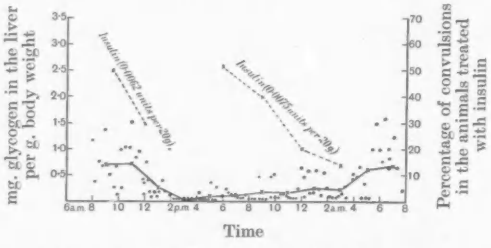

Fig. 3. Glycogen content of the liver of mice, which had fasted 10 hours before analysis.

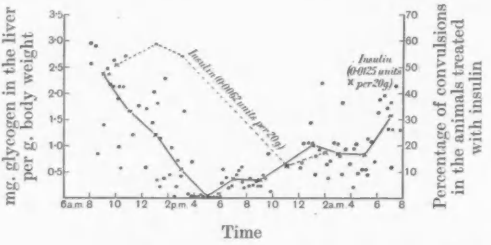

Fig. 4. Glycogen content of the liver of mice, which had fasted 10 hours before analysis.

12 midnight showed an average of 4·8 ± 0·681, the 50 killed between 12 midnight and 10 a.m. 8·6 ± 1·17. The figures are found in Table I, B. Figs. 3 and 4

give the glycogen content of the liver of mice, which had fasted 10 hours before killing. The same periodicity is shown in these figures, especially in Fig. 4. Here the average figure for the glycogen content of the liver of the 40 animals killed between 2 p.m. and 12 midnight was 0·42 ± 0·060 mg. per g. bodyweight, whereas the figure for the 70 animals killed between 8 a.m. and 2 p.m. and between 12 midnight and 8 a.m. was 1·32 ± 0·0867. The figures are given in Table I, C. The glycogen content of the livers of the 60 animals (see Fig. 3) killed between noon and midnight was 0·16 ± 0·078 and of the 57 killed between midnight and noon 0·53 ± 0·057 mg. per g. body weight.

The urinary nitrogen output was followed in three series with 10 rabbits in each. The animals were kept fasting for a period of 6 days, during which time the urine was collected quantitatively and analysed for nitrogen. Throughout all the series it could be established that the urinary nitrogen output was larger during the night than during the daytime. Owing to several circumstances the first two series yielded incomplete results. We therefore report only the third series (see Table II). In the second series the nitrogen output during

Table II. *Urinary nitrogen of rabbits excreted during periods of 12 hours.*

mg. nitrogen.

Rabbit	Time	Day of fasting 1	2	3	4	5	6
1	8.30 p.m.–8.30 a.m.	779	903	697	721	695	562
	8.30 a.m.–8.30 p.m.	655 +19	780 +16	591 +17	688 + 5	598 +15	481 +17
2	8.30 p.m.–8.30 a.m.	680	861	732	592	457	588
	8.30 a.m.–8.30 p.m.	868 −22	713 +21	532 +37	486 +22	512 −11	491 +20
3	8.30 p.m.–8.30 a.m.	778	865	773	628	685	750
	8.30 a.m.–8.30 p.m.	763 + 2	792 + 9	632 +22	580 + 8	717 − 4	669 +12
4	8.30 p.m.–8.30 a.m.	835	935	832	758	746	554
	8.30 a.m.–8.30 p.m.	798 + 5	819 +14	712 +17	616 +23	622 +20	487 +14
5	8.30 p.m.–8.30 a.m.	1005	1100	1043	632	578	623
	8.30 a.m.–8.30 p.m.	780 +29	895 +23	782 +33	552 +14	476 +21	438 +42
6	8.30 p.m.–8.30 a.m.	875	1010	.	812	654	600
	8.30 a.m.–8.30 p.m.	768 +14	840 +20	875	670 +21	578 +13	495 +21
7	8.30 p.m.–8.30 a.m.	698	817	820	674	572	485
	8.30 a.m.–8.30 p.m.	.	691 +18	669 +23	590 +14	509 +12	466 + 4
8	8.30 p.m.–8.30 a.m.	.	928	882	660	641	634
	8.30 a.m.–8.30 p.m.	673	823 +13	709 +24	618 + 7	588 + 9	565 +12
9	8.30 p.m.–8.30 a.m.	952	853	868	598	529	546
	8.30 a.m.–8.30 p.m.	626 +52	.	642 +35	542 +10	512 + 3	436 +25
10	8.30 p.m.–8.30 a.m.	840	753	855	.	718	696
	8.30 a.m.–8.30 p.m.	576 +46	728 + 3	714 +20	575	540 +33	640 + 9

the night was, on an average, 16 % larger than in the daytime for the first 3 days and 6·6 % larger during the last 3 days of the fasting. In the third series we succeeded in collecting the urine on each occasion except 6 out of a total of 120. The table shows a constantly increased output of nitrogen during the night, which persists in spite of the continued fasting.

Only on 3 occasions out of 55, on which the morning and the evening figures were recorded, did we observe a lower output of nitrogen in the

morning than in the evening. To judge by the evening figures, the low output on one of these occasions (rabbit No. 2, first day) was certainly due to a defective evacuation of the bladder in the morning, and on another occasion (the same rabbit on the 5th day) it was probably due to the same reason.

In the table the differences between the morning and the evening figures are given as percentages of the evening values. If we take the average for the positive figures, the increased output during the night amounts to 21·2 % for the first 3 days and to 15·8 % for the last 3.

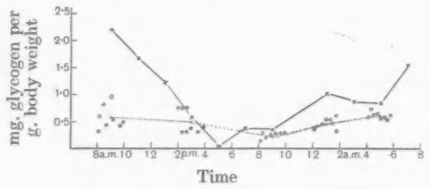

Fig. 5. Glycogen content of the muscular tissue (× ····· ×) of mice, which had fasted 10 hours before analysis, as compared with the glycogen content of the liver of the animals (× —— ×).

In the series recorded in the table the rabbits showed no rise of temperature and a moderate decrease of weight, so that the results were not distorted by any complications.

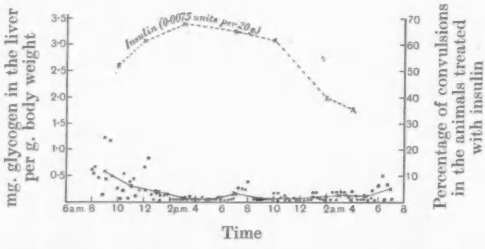

Fig. 6. Glycogen content of the liver of mice, which had fasted 10 hours before the experiment and were kept fasting.

It may be presumed that this increased nitrogen output during the night is genetically connected with the formation of glycogen. The cyclic accumulation of glycogen in the liver may be attributed partly to a conversion of proteins into glycogen. If we suppose that a rabbit excretes 1·5 g. nitrogen in 24 hours, the increased output during the night would be about 0·1–0·2 g., corresponding to approximately 0·4–1·0 g. glycogen. Thus in a rabbit's liver weighing 60–70 g. up to 1·5 % of glycogen, or possibly somewhat more, could be formed solely from the excess of amino-acids deaminised during the night. It seems reasonable to assume that the body proteins participate in this process, seeing that it is only slightly affected by fasting.

The excess output of nitrogen in these experiments, which is about 10 %

of the total amount of nitrogen eliminated, must represent the minimum amount of proteins thus converted into glycogen. It may, however, be presumed that the proteins metabolised are transformed into glycogen to a much larger extent than can be traced in this way.

Summary.

There are cyclic changes in the glycogen content of the liver of rabbits, rats and mice, which are to a large extent independent of the intake of food. Glycogen accumulates in the liver during the night and disappears again to some extent during the next morning. The same occurs even in fasting animals.

Similar periodical changes occur, though to a minor extent, in the muscles also.

As might have been expected from the observations recorded above, mice show an increased resistance to insulin in the afternoon and particularly during the night.

In rabbits, where these periodical changes are particularly striking, the urinary nitrogen output is increased during the night. In fasting rabbits the excess of nitrogen thus eliminated is about 20 % of the amount excreted during the day. This indicates that an increased deamination of amino-acids occurs during the night and that the formation of glycogen takes place partly at the expense of the body proteins.

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LXXXIX. METABOLISM STUDIES IN TETANY.

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A DISTURBANCE in chlorine metabolism has long been associated as an etiological factor in the production of certain forms of tetany. MacCallum and his co-workers [1920] attributed the onset of tetany in cases of pyloric obstruction to the loss of the gastric hydrochloric acid by the vomitus with the consequent production of an alkalosis. Grant [1922] found in one of six cases of adult tetany a marked reduction in the blood-chlorine. Iversen and Hansborg [1922] showed that after thyroidectomy sodium chloride was retained in the tissues to an abnormal extent although the amount of chlorine in the blood was reduced. Parhon, Ballif and Derevici [1926] also reported a fall in the serum-chlorine. Rockemann [1923] found that tetany following injection of sodium dihydrogen phosphate was accompanied by decreased excretion of urinary chloride.

The present investigation was undertaken primarily to study the changes in chlorine metabolism during conditions of tetany not associated with vomiting. Much work, however, has already been done indicating that in many forms of tetany there is a profound disturbance in calcium metabolism. Numerous experimental studies by Berkeley and Beebe [1909], Cooke [1911] and others showed that after parathyroidectomy there occurs a definite increase in the output of nitrogen, while Burns [1917] recorded a similar finding following guanidine injection. The first section of this paper accordingly deals with the effect of tetany on chlorine metabolism, while succeeding parts describe the alterations in nitrogen and mineral metabolism.

Methods.

Tetany was induced in cats by removal of the parathyroids and in rabbits by injection of guanidine. The parathyroids were first removed under chloroform anaesthesia; this usually produced a condition of latent tetany indicated by sluggishness and the other characteristic manifestations. A second operation of complete thyroidectomy led to the development of active tetany. The effect of guanidine was investigated by the subcutaneous injection of a 6 % solution of guanidine carbonate. 5 cc. were used in each case except rabbit 2

where the dose was 7 cc. An interval of 6 days elapsed between succeeding injections. The food was weighed and aliquot portions of each article of diet were analysed. The unused residues of the diet were kept, thoroughly mixed and analysed. Accordingly the intake during each period of the experiment was accurately known. The rabbits were fed on a mixture of equal parts of oats, bran and linseed, while the cats received oatmeal porridge and milk. Urine and faeces were collected separately. The urine was analysed daily while the faeces for each period were collected, dried on a steam-bath and analysed. The methods of analysis employed were: chloride [Van Slyke, 1923]; calcium [McCrudden, 1911]; phosphorus, Neumann, total nitrogen, Kjeldahl; purines, modified Camerer method [Cathcart *et al.*, 1925], creatine and creatinine, Folin; ammonia, Folin aeration method; amino-acids, Sørensen.

Chlorine.

Results.

A. *Urine* (Table I). It is clear that the urinary output of chlorine was diminished after removal of parathyroids or injection of guanidine much more than can be accounted for by the decreased intake. Accordingly there

Table I. *Showing intake, output and retention of chlorine in mg. daily.*

Cat	Period	Intake	Output	Re-tention	% re-tention
1	1. Normal	790·6	672·5	118·1	14·9
	2. Latent tetany	718·7	496·0	222·7	30·9
	3. Recovery	830·4	529·4	301·0	36·3
	4. Active tetany	194·2	Nil	194·2	100·0
	5. Recovery	706·0	1267·0	Negative	Negative
2	1. Normal	919·0	906·7	12·3	1·3
	2. Latent tetany	1000·0	642·0	358·0	35·8
3	1. Normal	707·4	583·6	123·8	17·5
	2. Latent tetany	528·0	157·4	370·6	70·1
	3. Active tetany	676·3	148·4	527·9	78·0
	4. Recovery	707·4	564·0	143·4	20·2
4	1. Normal	400·7	379·6	21·1	5·2
	2. Latent tetany	400·4	260·4	140·0	34·9
5	1. Normal	431·4	372·7	58·7	13·6
	2. Latent tetany	430·0	287·0	143·0	33·2
Rabbit					
1	1. Normal—7 days	30·6	30·1	0·5	1·63
	2. Guanidine—5 days following 1st injection	18·2	8·6	9·6	52·7
	3. Guanidine—5 days following 2nd injection	8·55	3·0	5·55	64·9
	4. Guanidine—10 days following 3rd injection	7·1	1·4	5·7	80·2
2	1. Normal—7 days	28·4	31·3	12·9	—
	2. Guanidine—5 days after 1st injection ...	7·1	4·04	3·06	43·1
3	1. Normal—7 days	42·6	41·2	1·4	3·3
	2. Guanidine—5 days following 1st injection	16·05	13·33	2·72	16·9
	3. Guanidine—5 days following 2nd injection	10·2	4·76	5·44	53·3
	4. Guanidine—10 days following 3rd injection	14·2	5·1	9·1	64·1
4	1. Normal—7 days	44·4	42·9	1·5	3·4
	2. Guanidine—5 days following 1st injection	8·66	4·54	4·12	47·5
	3. Guanidine—5 days following 2nd injection	7·1	2·44	4·66	65·6
	4. Guanidine—10 days following 3rd injection	8·6	2·40	6·20	72·1

was actually an increased retention of chlorine during both latent and active tetany periods although the intake was in most cases much less than that of the normal. If sufficient chlorine had been ingested the retention was more marked in the active stage.

B. *Blood* (Table II). The immediate effect of an injection of guanidine on the total chlorine content of the blood was with one exception (rabbit 11) a reduction of 8 to 18 milli-equiv. per litre. This reduction was followed within

Table II. *Showing total and volatile chlorine of blood in normal condition and active and latent tetany (m.-eq. per litre).*

	Cat 1		Cat 3		Cat 5		Cat 6		Cat 7		Cat 8	
Period	Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl
Normal	102·6	14·6	101·8	11·4	96·2	10·4	98·6	11·8	104·7	12·2	98·0	10·0
	—	—	100·4	10·4	97·1	11·2	100·1	11·8	106·0	12·0	100·2	10·4
	—	—	—	—	—	—	—	—	106·2	11·7	100·3	10·5
Latent tetany	—	—	—	—	—	—	91·2	12·9	93·1	16·3	92·5	12·1
	122·1	19·8	—	—	109·5	20·4	110·5	18·1	113·2	17·4	112·6	14·6
	—	—	—	—	108·7	18·3	112·8	20·6	115·9	17·3	115·9	19·8
	—	—	—	—	110·5	12·3	112·8	20·3	113·8	20·0	115·4	19·2
Active tetany	—	—	—	—	110·0	0·2	—	—	102·8	0·0	107·5	1·0
Recovery	105·8	12·6	103·0	11·6	—	—	—	—	—	—	—	—

	Rabbit 1		Rabbit 2		Rabbit 3		Rabbit 4		Rabbit 5		Rabbit 6	
Normal	80·2	8·6	74·6	12·2	85·0	8·6	77·6	11·2	82·5	10·0	67·3	11·2
	80·0	10·2	75·1	12·6	84·8	10·4	78·9	10·8	80·6	10·0	67·4	11·5
1st day after 1st injection	70·3	0·0	75·5	0·7	76·8	0·8	62·5	3·6	70·3	2·1	50·8	3·5
4th day after 1st injection	81·8	17·4	80·4	15·3	88·2	19·3	80·9	20·0	80·6	17·8	75·5	16·3
1st day after 2nd injection	72·9	−0·1	—	—	75·8	2·3	70·7	2·2	68·9	4·5	58·8	5·1
4th day after 2nd injection	86·6	17·7	—	—	80·4	19·6	86·5	18·6	78·8	18·4	75·9	16·1
1st day after 3rd injection	78·8	−0·2	—	—	75·9	1·1	80·4	4·9	75·5	0·3	70·2	0·3
8th day after 3rd injection	73·0	12·6	—	—	73·0	9·6	78·1	13·3	67·7	7·2	75·5	10·7

3 to 4 days by a return to a normal or slightly super-normal figure except in the period following the third injection. In two cases after the third injection a return to normal was recorded while in the other three the total chlorine was still further reduced. The volatile chlorine of the blood almost vanished immediately after administration of guanidine, only to return 4 days after the injection to a value exceeding the normal figure.

Similar results were noted following the removal of parathyroids. The onset of latent tetany was accompanied by a decrease of the total chlorine of the blood with a rise in the volatile portion. As the state of latent tetany was prolonged the total chlorine rose to a value 10–15 % in excess of the normal, while the volatile chlorine became almost twice as great as in health. When active tetany was induced by a complete thyreo-parathyroidectomy the total chlorine still remained above normal in two of the three cases, while

the volatile form almost completely disappeared. In two cats in which as the result of treatment with saline there were no evident symptoms the blood-chlorine values were normal.

C. *Tissues* (Table III). Analysis of the tissues showed that in both the latent and active stages of tetany due either to parathyroidectomy or guanidine poisoning there occurred a marked increase of the total Cl of muscle, heart

Table III. *Showing total and volatile chlorine of tissues in normal condition and active and latent tetany (m.-eq. per litre).*

		Muscle		Heart		Liver		Kidney		Lung	
Cat	Condition	Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl
A	Normal	15·0	1·8	32·1	6·1	27·2	3·4	41·0	7·0	61·1	11·1
B	,,	13·6	4·1	—	—	26·3	7·8	40·0	4·4	—	—
C	,,	14·2	2·2	26·6	6·2	31·5	2·3	35·0	5·0	62·5	11·7
	Average	14·3	2·7	29·4	6·2	28·3	4·5	38·7	5·5	61·8	11·4
6	Latent tetany	51·0	28·2	67·0	36·4	34·5	19·7	51·2	9·2	57·7	22·0
2	Active tetany	63·6	2·1	77·8	0·0	62·2	0·1	56·3	0·0	42·0	0·2
5	,,	63·8	3·1	86·0	0·7	66·0	0·0	63·3	0·0	51·2	0·0
7	,,	54·0	0·2	85·0	0·1	40·0	1·7	64·0	0·0	57·2	1·1
8	,,	76·5	0·7	48·4	0·4	60·4	0·2	40·8	0·8	55·9	2·9
	Average	65·0	1·5	74·3	0·3	57·2	0·5	56·1	0·2	51·6	1·1
1	Recovered	46·0	5·6	50·5	20·4	28·2	5·8	50·0	5·0	54·0	4·9
3	,,	23·0	3·0	40·0	16·0	30·0	5·2	53·0	4·0	51·0	21·4
	Average	34·5	4·3	45·3	18·2	29·1	5·5	51·5	4·5	52·5	13·2
Rabbit											
7	Normal	7·3	3·7	34·2	9·9	27·6	3·5	—	—	48·8	16·2
8	,,	11·0	3·9	36·0	9·6	22·0	2·0	48·6	14·1	50·0	15·9
9	,,	15·6	4·7	34·8	8·5	23·0	5·1	53·8	19·6	49·2	13·0
	Average	11·3	4·1	35·0	9·3	24·2	3·5	50·7	16·9	49·3	15·0
3	Latent tetany	35·0	25·2	55·0	12·5	39·5	24·3	37·8	10·4	43·5	14·7
4	,,	31·0	19·9	52·0	7·0	41·4	14·9	44·0	9·9	41·4	11·6
5	,,	41·0	32·8	55·4	12·2	42·4	24·1	43·2	25·6	47·7	17·7
	Average	35·7	26·0	54·1	10·6	41·1	21·1	41·7	15·3	44·2	14·7
1	Active tetany	46·0	0·2	54·6	0·6	40·1	1·5	23·7	0·8	46·3	0·3
2	,,	42·4	0·5	56·4	0·4	38·9	0·1	29·8	1·0	47·2	0·4
6	,,	40·0	2·8	55·7	1·9	37·8	1·8	28·5	1·4	41·2	1·2
	Average	42·8	1·2	55·6	1·0	38·9	1·1	27·3	1·1	44·9	0·6

and liver. Following injection of guanidine the total Cl of kidney and lung was reduced, while after removal of the parathyroids the Cl was increased in the kidney but reduced in the lung. During the latent tetany stage the volatile Cl was increased markedly in muscle and liver and slightly in kidney in both series of experiments. In heart and lung the volatile portion was unchanged in the guanidine-treated group but definitely increased in the parathyroidectomy series. During active tetany in both groups there was almost complete disappearance of volatile Cl. Two cats, which were killed during a period of freedom from all manifest signs of tetany, showed a moderate increase in the total Cl content of heart and skeletal muscle but otherwise practically normal values.

Discussion.

Guanidine. From these results it is permissible to conclude that guanidine poisoning creates a demand for chlorine in the tissues. The early fall in the total chlorine content of the blood coupled with the diminution in the urinary excretion is clearly a consequence of the withdrawal of chlorine to the tissues. The subsequent rise of the blood-chlorine is in all probability the result of gradual repletion of the blood from the chlorine ingested.

It has already been shown that chlorine exists in part as a volatile organic compound which has the power of forming a complex with substances containing the amino-group [Morris and Morris, 1930]. When so combined the power of volatilisation at 100° is lost. The amount of non-volatile amino-chlorine complex depends on the proportion of volatile organic chlorine to amino-groups, closely following the adsorption formula. The changes in the content of volatile chlorine of blood and tissues are strongly suggestive of the formation of amino-compounds in the tissues. Within 24 hours of the injection of guanidine the volatile chlorine of the blood has completely disappeared while the non-volatile fraction remains practically unaltered in amount. As there is no excessive excretion of chlorine it must be concluded that the volatile chlorine has gone to the tissues. Four days after the injection the volatile chlorine in the blood has increased above its normal value. Meanwhile in order to satisfy the extra demands there is an excessive retention of the chlorine ingested. The results of tissue analysis during the latent tetany stage conform with this view. It is seen that during that period there is a great increase in the total chlorine, affecting in muscle and liver only the volatile form. Renal tissue, however, contains rather less chlorine in this period, the result probably of less chlorine having been carried to the kidney for excretion. It is possible that the increase of volatile chlorine is due to the presence of guanidine itself. If that were so it would be reasonable to expect either that it should be more or less uniformly distributed over the tissues or that excess should be found only in the liver, the chief seat of deamination. It is significant, however, that skeletal muscle, one of the chief centres of metabolic activity, shows equally with liver a marked increase in the amount of volatile chlorine.

In the stage of active tetany the striking feature of the results of tissue analysis is the great diminution in volatile chlorine, which must be due to the presence of excessive amount of amino-compound. The total chlorine of all the tissues except kidney remains high as in the latent tetany stage.

Parathyroidectomy. The results show that chlorine metabolism was affected in almost the same manner as in guanidine poisoning In several instances intraperitoneal injection of sodium chloride led to a rapid disappearance of the symptoms. This was shown by Paton and Findlay [1917] who attributed the beneficial action of the saline to a dilution of the poison. It seems from the present study more correct to refer the effect of administered

chloride to a neutralisation of the protein breakdown product by the formation of an amino-chlorine complex.

It appears justifiable, therefore, to conclude that the production of tetany by guanidine poisoning or by parathyroidectomy is the result of excessive proteolysis leading to the liberation of a large amount of amino-nitrogen which unites with the volatile chlorine. As long as the ratio of volatile chlorine to amino-nitrogen is maintained above a certain value manifest symptoms of tetany do not appear. When the liberation of amino-nitrogen proceeds apace and reduces this ratio the volatile chlorine disappears owing to the formation of a non-volatile complex, and active tetany ensues. Should the ratio again be raised by the parenteral administration of chlorine, owing to the rapid formation of volatile chlorine the symptoms again become latent. It is well known that the feeding of meat hastens the onset of tetany, a result to be expected in view of the increased amount of circulating amino-nitrogen. This view would explain the discordant results recorded of guanidine poisoning. Obviously the production of manifest tetany would depend on two factors, (*a*) the extent of protein breakdown and (*b*) the available supply of chlorine. The greater the supply of chlorine the larger would be the dose of guanidine necessary to produce symptoms. Further, the guanidine having itself an amino-group might conceivably be detoxicated before it could act on the tissues, provided there was available an abundance of chlorine.

Still further support is lent to our hypothesis by a consideration of gastric tetany. In this form there is a great loss of chlorine by the vomitus leading to a great deficiency in the tissues, which coincides with normal or slightly excessive liberation of amino-nitrogen. Further, it is of interest to state that in the idiopathic tetany of children there also occurs a marked retention of chlorine [Morris and MacRae, 1931].

Nitrogen.

The results noted in Table IV indicate that, following parathyroidectomy, in spite of decreased intake the urinary output of nitrogen was markedly increased so that the retention was greatly diminished, there being in four instances a net loss. The faecal nitrogen content varied but little. This is in accord with the numerous experimental studies previously recorded by Berkeley and Beebe [1909] and others for tetania parathyreopriva and by Burns [1917] for guanidine tetany. The partition of nitrogen in the urine is of importance in indicating the source of the excess nitrogen. The results heretofore recorded in the literature are conflicting. Berkeley and Beebe [1909] found an increase in the ammonia of the blood and urine and because of this and of the effectiveness of calcium therapy in parathyroid tetany and ammonia intoxication concluded that the excess of ammonia was the causal agent in the production of tetany. Falta and Kahn [1912] and Underhill *et al.* [1922] also found an increase in ammonia whereas Carlson and Jacobson [1911], Greenwald [1913], Albertoni [1914] could only show a slight rise in the ammonia

Table IV. *Showing intake, output and retention of nitrogen in g. daily.*

Cat	Period	Intake	Output			Retention	NH_3	Total* creatinine	Amino-* acid-N	Purine*-N
			Urine	Faeces	Total					
1	Normal	3·5200	1·2400	1·6209	2·8600	0·6592	—	167·40	101·52	3·478
	Latent	3·2700	1·9725	1·7100	3·6825	0·4125	—	50·20	359·56	31·500
	Active	0·7205	1·5170	0·0000	1·5170	0·7965	—	70·70	203·60	30·080
	Recovery	2·9100	0·8824	0·6911	1·5174	1·3926	—	124·40	172·02	3·500
2	Normal	5·7370	1·7130	3·5400	5·2530	0·4840	—	234·36	113·40	6·456
	Latent	5·7000	2·8180	3·7610	6·5790	0·8790	—	66·75	344·25	20·410
3	Normal	3·9000	0·6886	0·2781	0·9667	2·9333	0·1294	137·06	63·95	2·514
	Latent	1·3730	0·8770	0·0370	0·9140	0·4590	0·0346	39·36	301·25	37·829
	Active	0·7080	2·1443	0·0000	2·1443	1·4363	0·0239	53·96	302·00	105·148
	Recovery	3·9000	0·6540	0·2581	0·9121	2·9879	0·0681	110·92	62·83	9·201
4	Normal	3·5300	0·5960	0·2820	0·8780	2·6520	0·1074	220·32	43·38	1·538
	Latent	2·2800	0·8430	0·1280	0·9710	1·3090	0·0507	135·66	47·54	41·025
5	Normal	2·6140	0·7306	0·0900	0·8206	1·7934	0·1279	243·00	70·65	1·032
	Latent	2·0500	0·9512	0·0821	1·0333	1·0167	0·0515	157·00	60·13	44·600
Rabbit										
1	Normal	1·4301	0·7767	0·3240	1·1007	0·3294	0·0296	24·50	61·30	9·87
	1st injection	0·7322	0·3986	0·3329	0·7315	0·0007	0·0165	25·10	38·60	11·80
	2nd ,,	0·3000	0·2310	0·2225	0·4535	−0·1535	0·0014	4·90	82·10	31·72
	3rd ,,	0·2860	0·3137	0·2005	0·5142	−0·2282	0·0004	—	131·40	33·87
2	Normal	1·1072	0·5997	0·2490	0·8487	0·2585	0·0370	24·50	22·00	12·04
	1st injection	0·2860	0·2460	0·1380	0·3840	−0·0980	0·0086	25·00	53·40	12·01
3	Normal	1·7875	0·8857	0·6000	1·4857	0·3018	0·0096	17·46	27·75	12·90
	1st injection	0·6464	0·3852	0·2082	0·5934	0·0530	0·0076	18·05	17·58	10·20
	2nd ,,	0·4118	0·3148	0·1071	0·4219	−0·0101	0·0018	5·18	41·84	30·70
	3rd ,,	0·5720	0·5839	0·1109	0·6948	−0·1228	0·0008	—	174·40	25·40
4	Normal	1·8000	0·8420	0·5850	1·4270	0·3730	0·0113	13·53	20·00	9·87
	1st injection	0·3489	0·1870	0·1800	0·3670	−0·0181	0·0123	13·00	16·08	10·00
	2nd ,,	0·3000	0·3201	0·1075	0·4276	−0·1276	0·0068	5·80	81·00	24·27
	3rd ,,	0·3000	0·4333	0·1069	0·5402	−0·2402	0·0016	—	152·80	22·89

* The figures for creatinine, amino-acid-N and purine-N are in mg. daily.

excreted in the urine and no excess in the blood. Carlson and Jacobson [1911] also showed that there is a difference in the therapeutic actions of calcium salts in ammonia poisoning and tetany. Wilson, Stearns and Thurlow [1915] found a lowering of the ammonia coefficient during latent, but a rise during active tetany. MacCallum and Voegtlin [1911] on the other hand obtained a definite increase immediately after parathyroidectomy. Cooke [1910] and Palladin and Grilliches [1924] reported in tetania parathyreopriva an increase in the total creatinine output whereas Burns [1917] obtained the opposite result after parathyroidectomy but a slight increase in guanidine tetany. Esau and Stoland [1930] concluded that the initial effect of parathyroidectomy is on tissue metabolism, the substance chiefly affected being phosphagen. Justschenko [1913] found an increase in the output of amino-acids, while Falta and Kahn's [1912] results indicated no change in the excretion of amino-acids but a rise in the peptide-nitrogen of the urine. Burns [1917] reported a slight rise in the output of amino-acids after one injection of guanidine.

Our results demonstrate the following points.

Total creatinine. After removal of the parathyroids there was a definite decrease in the amount of total creatinine during latent and active tetany with a tendency to return to normal in the recovery stage. After the first

injection of guanidine, however, the output was unchanged or slightly increased but following the later injections it was markedly diminished. After the third injection creatinine could not be detected in the urine.

Ammonia. The amount excreted was diminished, the decrease becoming more marked as the tetany became more manifest.

Amino-acids. After parathyroidectomy the amino-acid content of the urine was markedly increased in all the tetany periods except two (in one it was practically unchanged and in the other somewhat reduced). Following the first injection of guanidine there occurred except in rabbit 2 a decrease in the amount of amino-acid excreted. The later injections of guanidine led to a marked increase in the output.

Purine-nitrogen. In all cases the output of purine-nitrogen was definitely increased.

From these results it seems fair to conclude that an immediate and important effect of parathyroidectomy and guanidine poisoning is an excessive tissue catabolism. It also seems that the effect on nitrogen metabolism is roughly parallel with the degree of tetany produced. This is more apparent in the guanidine series where it is evident that the effect of guanidine is dependent on the dosage. The second and third injections produced more marked effects.

Minerals.

It is clear that comparison between the retentions of calcium and phosphorus in the various periods is difficult owing to the difference in intakes. Accordingly we have estimated the retention as a percentage of the intake. In calculating the difference in the amount of calcium or phosphorus excreted we have calculated the expected excretion of the normal period on the same calcium or phosphorus intake as that of the tetany period. This admittedly is not satisfactory, but the fact that we obtain such close correlation between the excess calcium calculated on this basis and the excess amino-acids seems to afford some justification for this method of computation. The only other course is to withhold food entirely during all the periods. Such a procedure is open to the very serious objection that ketosis with all its concomitant metabolic effects complicates the picture.

Calcium.

Our results (Table V) may be summarised as follows.

After removal of the parathyroids the urinary output of calcium was increased in the stage of latent tetany and still more in the active period, the faecal output was decreased but not markedly so and the retention of calcium calculated as a percentage of the intake was decreased.

After the first injection of guanidine there occurred a decrease of urinary calcium. The second and still more the third administration of guanidine led to a rise in the amount of calcium in the urine. The percentage retention was

Table V. *Showing intake, output and retention of calcium and phosphorus in g. daily.*

		Calcium						Phosphorus					
			Output			Retention			Output			Retention	
Cat	Period	Intake	Urine	Faeces	Total	Total	%	Intake	Urine	Faeces	Total	Total	%
1	Normal	0·3309	0·0311	0·1034	0·1345	0·1964	59·3	0·4107	0·1545	0·1325	0·2870	0·1237	30·1
	Latent	0·2512	0·0388	0·0878	0·1266	0·1246	49·6	0·3678	0·1667	0·0876	0·2543	0·1135	30·8
	Active	0·0765	0·0410	0·0000	0·0410	0·0355	46·4	0·1824	0·1210	0·0000	0·1210	0·0614	33·9
	Recovery	0·1960	0·0354	0·0512	0·0866	0·1094	55·9	0·1900	0·0800	0·0438	0·1238	0·0662	82·7
2	Normal	0·3417	0·0620	0·1450	0·2070	0·1347	39·4	0·4715	0·2017	0·2200	0·4217	0·0498	24·7
	Latent	0·3635	0·1480	0·1076	0·2556	0·1079	29·7	0·4300	0·2250	0·1302	0·3552	0·0748	33·2
3	Normal	0·2030	0·0140	0·1322	0·1462	0·0568	28·0	0·2933	0·1090	0·1972	−0·3063	−0·0130	—
	Latent	0·1270	0·0113	0·1021	0·1134	0·0136	10·9	0·1530	0·0792	0·0831	0·1623	−0·0093	—
	Active	0·0570	0·0640	0·0000	0·0640	−0·0070	—	0·1100	0·1628	0·0000	0·1628	−0·0528	—
	Recovery	0·2030	0·0100	0·1411	0·1511	0·0519	25·0	0·2933	0·0705	0·2324	0·3029	−0·0096	—
4	Normal	0·2010	0·0068	0·1021	0·1089	0·0921	45·8	0·2830	0·0925	0·1001	0·1926	0·0904	31·9
	Latent	0·1650	0·0113	0·0786	0·0899	0·0751	45·5	0·2500	0·1074	0·0625	0·1699	0·0801	32·0
5	Normal	0·1690	0·0089	0·0885	0·0974	0·0716	42·4	0·2280	0·0935	0·0870	0·1805	0·0475	20·8
	Latent	0·1350	0·0085	0·0614	0·0699	0·0651	48·2	0·1650	0·0910	0·0390	0·1300	0·0350	21·2
Rabbit													
1	Normal	0·0904	0·0094	0·0304	0·0398	0·0506	56·0	0·9010	0·5737	0·1555	0·7292	0·1718	19·0
	1st injection	0·0474	0·0046	0·0151	0·0197	0·0277	58·4	0·4500	0·2870	0·0780	0·3650	0·0850	18·9
	2nd ,,	0·0242	0·0041	0·0085	0·0126	0·0116	47·9	0·2002	0·1403	0·0295	0·1698	0·0304	15·2
	3rd ,,	0·0185	0·0092	0·0061	0·0153	0·0032	17·3	0·1802	0·1413	0·0188	0·1601	0·0201	11·1
2	Normal	0·0700	0·0073	0·0236	0·0309	0·0391	55·8	0·6660	0·3000	0·1270	0·4270	0·2390	35·9
	1st injection	0·0201	0·0022	0·0070	0·0092	0·0109	54·2	0·1800	0·1168	0·0464	0·1182	0·0618	34·3
3	Normal	0·1140	0·0120	0·0401	0·0521	0·0619	45·7	1·1340	0·7632	0·2195	0·9827	0·1513	13·3
	1st injection	0·0380	0·0039	0·0128	0·0167	0·0213	56·0	0·3868	0·2608	0·0740	0·3348	0·0480	12·4
	2nd ,,	0·0282	0·0047	0·0096	0·0143	0·0139	49·3	0·2600	0·2015	0·0325	0·2340	0·0260	10·0
	3rd ,,	0·0370	0·0201	0·0122	0·0323	0·0047	12·6	0·3600	0·2888	0·0408	0·3296	0·0304	8·4
4	Normal	0·1135	0·0289	0·0305	0·0594	0·0541	47·6	1·1409	0·7856	0·1740	0·9596	0·1813	15·9
	1st injection	0·0224	0·0060	0·0054	0·0014	0·0110	49·0	0·2196	0·1380	0·0444	0·1824	0·0462	21·0
	2nd ,,	0·0242	0·0118	0·0070	0·0188	0·0054	22·3	0·2002	0·1478	0·0286	0·1764	0·0238	11·9
	3rd ,,	0·0242	0·0181	0·0066	0·0247	−0·0005	—	0·2002	0·1563	0·0205	0·1768	0·0234	11·7

unchanged or slightly increased after the first injection of guanidine but diminished after the second and third doses.

Cybulski [1906], Haskins and Gerstenberger [1909] and Salvesen [1923] found a decreased retention of calcium in tetany, the increased output being both by urine and faeces. Cooke [1910] failed to obtain an increase of calcium either in the urine or faeces and maintained that tetany was produced by an altered salt equilibrium brought about by accumulation of acid. Greenwald [1929] although he upholds the low calcium theory of the causation of tetany, found with Hastings and Murray [1921] a retention of calcium. Several investigators have mentioned the possibility of acid substances being involved in the increased loss of lime. Thus Salvesen attributed the loss of lime to the high phosphate content of the blood. Table VI shows the close relationship between the excess excretion of total calcium and that of amino-acids. The excess amino-acid/excess calcium ratio in the parathyroidectomy series varied from 10·3 to 10·8 in all but one instance and in all but two of the guanidine series from 9·5 to 11·0. In the exceptions it will be noted that there was an actual increase in the retention of calcium and a reduction in the output of amino-acids. The other results which show such a close correlation, seem to

Table VI. *Calcium and amino-acid excretion.*

Cat	Period	Excess Ca over normal excretion in g.	Excess amino-acid over normal excretion in g.	Amino-acid Calcium
1	Latent	0·0245	0·25804	10·53
	Active	0·0099	0·10210	10·31
2	Latent	0·0220	0·23805	10·82
3	Latent	0·0220	0·23730	10·78
	Active	0·0230	0·23805	10·35
4	Latent	0·0004	0·00416	10·4
5	Latent	−0·0080 (retention)	−0·01052 (retention)	1·32
Rabbit				
1	1st injection	−0·0058	−0·0227	3·91
	2nd ,,	0·0020	0·0208	10·4
	3rd ,,	0·0071	0·0701	9·9
2	1st ,,	0·0031	0·0314	10·1
3	1st ,,	−0·0007	−0·01017	14·5
	2nd ,,	0·0014	0·01409	10·6
	3rd ,,	0·0154	0·14665	9·5
4	1st ,,	−0·0004	−0·00392	9·8
	2nd ,,	0·0061	0·06100	10·0
	3rd ,,	0·0120	0·13280	11·0

indicate that there is some etiological relationship between the excess production of amino-acids and the decreased retention of calcium. These findings lead one tentatively to suggest that the decalcification of the bones which has been shown radiologically to occur after parathyroidectomy [Erdheim, 1911] may be due to the formation of amino-acids in excessive amounts.

Phosphorus.

Greenwald [1922] found a decreased retention of phosphorus during the latent stage following parathyroidectomy and a marked increase during the active period. Recently Greenwald [1929] concluded that the only two constant changes following parathyroidectomy were a fall in serum-calcium and a retention of phosphorus and maintained that the phosphorus is precipitated in the tissues as calcium phosphate. The increase in blood-phosphorus he attributed to a breakdown of lipoids and proteins containing phosphorus. Esau and Stoland [1930] have elaborated this theory on the basis of the recent work carried out on the relationship of phosphocreatine to muscle metabolism.

The results in connection with phosphorus metabolism are given in Table V. They indicate that after parathyroidectomy there is but slight change in the retention of phosphorus. After injection of guanidine the percentage retention of phosphorus is diminished, the diminution becoming more marked after succeeding injections. Owing to the marked reduction in the intake it may be urged that the decrease in the percentage is a result not of abnormal metabolic processes but simply of the smaller amounts ingested. In rabbit No. 4, however, the intake of phosphorus was increased in the periods following the second and third injections and despite this the absolute amount of phosphorus retained was appreciably less. This lends support to the

view that there is an increased catabolism of phosphorus-containing substances as a result of guanidine poisoning.

In view of the diminished retention of calcium and the fact that four-fifths of the calcium of the body is united with phosphorus it seems at first glance strange that the phosphorus retention should not run parallel with that of calcium. It has to be remembered, however, that whereas practically the sole depôt of calcium is bony tissue, considerable amounts of phosphorus are also found in combination with lipoids and nitrogenous substances.

On the basis of the results recorded here it seems possible to formulate a hypothesis as to the chemical pathogenesis of tetany produced either by parathyroidectomy or by injection of guanidine. The primary effect in both cases is to produce an abnormal protein catabolism resulting in the formation of some toxic amino-compound which immediately combines with an organic chlorine compound. Simultaneously there occurs an excessive catabolism of phosphorus-containing substances and formation of large amounts of amino-acids. The effect on calcium and phosphorus suggests that the excess amino-acid carries away calcium from the bone leaving phosphorus either to be excreted or to form a phosphocreatine compound. Whether the extra phosphocreatine formed in the body is the result of excessive guanidine formation following parathyroidectomy we have no means of deciding from the data presented here. It is certainly suggestive that injection of guanidine produces metabolic results bearing a striking resemblance to those following removal of the parathyroids.

There is not the same gradation in the results observed following guanidine injection as there is after parathyroidectomy. It must be remembered, however, that in the latter case the abnormality produced leads either to continuous production of some abnormal substance or to an equally continuous process of excessive protein breakdown. In guanidine poisoning under the conditions of the experiment there is but one massive injection of poison. If this dose is too great death is likely to ensue, whereas if the body is able to deal with it a return to normal is likely to follow provided a second injection is not given too quickly. The fact that chlorine is retained in such large amount after even a single injection of guanidine, and this despite the fact that there is comparatively little upset of nitrogen metabolism, suggests that guanidine is detoxicated by a chlorine compound. If the available chlorine is sufficient to detoxicate the guanidine there will be but little disturbance. This would explain the variability in the results recorded of guanidine injection. The immediate effect at any rate seems to depend not simply on the amount of guanidine injected but on the amount of available chlorine. The fact that succeeding doses of guanidine of the same amount as the first produce effects quite out of proportion to the first indicates that the amount of available chlorine progressively decreases.

The actual onset of active tetany, if this hypothesis be correct, does not occur until the amount of toxic substance exceeds the amount of available

chlorine. As already mentioned this would explain the conditions of so-called tetania chloropriva. Furthermore, the carrying away of the calcium by the excess amino-acids would account for the low serum-calcium. The rise in inorganic phosphorus which tends to occur could be explained by the extra production of acid-soluble phosphorus resulting from abnormal nucleoprotein catabolism.

Summary.

Following parathyroidectomy or injection of guanidine there occur the following metabolic disturbances.

A. *Chlorine.* There is a marked retention of chlorine in the tissues. In the latent tetany stage there is an increase of volatile chlorine both in the blood and tissues while in the active period there is almost complete absence of volatile chlorine.

B. *Nitrogen.* There is an increased catabolism of tissue protein associated with a rise in the urinary output of total nitrogen, amino-acids and purine-nitrogen and a fall in creatinine and ammonia.

C. *Calcium.* The percentage retention is decreased as a result of relative excess in the urinary excretion. There is a correlation between the excess of calcium and amino-acids excreted.

D. *Phosphorus.* The retention is either slightly diminished or unchanged.

It is suggested (1) that the primary effect in both parathyroidectomy and guanidine poisoning is an abnormal breakdown of tissue proteins and that the changes in calcium, phosphorus and chlorine are secondary to the disturbance in nitrogen metabolism, and (2) that the appearance of active tetany is in part at any rate due to a relative deficiency of chlorine compared with some toxic amino-compound occurring in the course of protein breakdown.

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XC. THE ENZYMIC HYDROLYSIS OF LECITHIN.

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Since the original isolation by Thudichum [1874] of lecithin from brain tissue, the study of this substance in relation to its chemistry and to its physiological significance has been a subject of increasing interest. The work of Willstätter and Lüdecke [1904], of MacLean [1912, 1915] and of Levene and his co-workers [1918, 1921, 1922, 1927] on the nature of its cleavage products on hydrolysis and on the preparation of the phosphatide in a pure state has thrown much light on the chemical nature of lecithin. Within recent years Grün and Limpächer [1927] have succeeded in synthesising, from distearin, phosphoric anhydride and choline, interesting substances, which, if not identical with the naturally occurring phosphatides, are certainly remarkably like them in their physical and chemical properties.

The rôle of phosphatides in metabolism has been a matter of considerable speculation and much experiment. McCollum *et al.* [1912] have shown that the nature of the fatty acid radicals in the lecithin of eggs changes with, and is presumably dependent on, the nature of the fats contained in the diet. Levene and Simms [1921] have shown that, as in the case of the fats themselves, the fatty acid radicals of the lecithin isolated from liver are more unsaturated than those of lecithin from brain or eggs. The view has been repeatedly advanced that the phosphatides play an important part in the metabolism of the fats as a whole. The fact that lecithin is more readily oxidised and hydrolysed than neutral fat and that the lecithin-fat of the liver, where desaturation is thought to take place, is more unsaturated than the fat of other parts of the body, may be taken as evidence supporting this view. Bloor and Knudson [1916, 1917] have observed a definite increase in the phosphatide content of both corpuscles and plasma following the ingestion of a meal containing fat, and Sinclair [1929] has found that the ingestion of cod-liver oil increases the iodine value of the lipoid fatty acids of the mucosa.

The importance of phosphatides as essential fat is well illustrated in the brain. This organ which contains the largest amount of phosphatide loses none of it during starvation, and the inference that this form of fat is essential to the life of the organ would seem justifiable.

The rôle of the body enzymes in relation to lecithin has received siderable attention from various workers. Bokay [1877] investigatec action of the intestinal lipase, and since then many publications dealing the hydrolysis of lecithin by intestinal and ricinus lipase have appea Some of the more recent work in this connection is due to Contardi and Latzer [1927], who studied the hydrolysis of lecithin cadmium chlorides, and by Paal [1929] who found the lipase cleavage of lecithin to give parallel results for the liberation of fatty acid and choline.

Since the study of body phosphatases during recent years has led to such important results in the elucidation of bone formation [Robison, 1923; Robison and Soames, 1924, 1925; Martland and Robison, 1926, 1927] and other physiological processes in health and disease [Kay, 1926, 1928, 1930; Neuberg, 1918, 1926, 1928; Meyerhof, 1928], it seemed possible that a study of the action of an enzyme which would hydrolyse lecithin to liberate phosphate might lead to a better understanding both of the chemical nature of this substance and of its significance in the body processes.

Kay [1926] noted the presence of a feeble "lecithinase" in an extract of kidney, which slowly hydrolysed phosphate from lecithin at 37° and had an optimum p_H of 7·4, about that of the tissues. He regards this as being a different enzyme from kidney phosphatase, the optimum p_H of which is 8·9, and which he has some experimental evidence to show is identical with intestinal and bone phosphatase. The latter Martland and Robison [1926] state to be without action on phosphatides.

The action of a phosphate-liberating enzyme on lecithin is more complex than that of the bone phosphatase on hexose- or glycero-phosphate, since it involves the cleavage of both choline and the glycerol residue. This, however, might be said of the enzymic hydrolysis of any secondary ester of phosphoric acid where two esterified groups must be separated in order to liberate free phosphoric acid; and the optimum p_H for hydrolysis of such esters, 7·7, as found by Asakawa [1929][1], is remarkably near that reported in a former investigation [King and Page, 1930] for the enzyme contained in extracts of intestine and kidney which hydrolysed lecithin most actively at p_H 7·5–7·6. This question of the relation of the enzymes responsible for the hydrolysis of primary and secondary esters of phosphoric acid and of lecithin will be dealt with more fully in a later communication. The investigation detailed in this paper consists of a study of the liberation of phosphoric acid from lecithin under various conditions and of the occurrence and distribution of the enzyme responsible for this hydrolysis in a large number of animal tissues.

Experimental.

That there is an enzyme present in autolysing tissues which is responsible for the liberation of free phosphate from compounds other than those usually

[1] Asakawa investigated the action of kidney phosphatase on a large number of primary and secondary esters of phosphoric acid, including diphenyl, dicresyl, diglyceryl and diethyl phosphate.

ed to as "acid-soluble" may be inferred from the fact that the "free hate" in a trichloroacetic acid extract of a tissue made after a few days' ysis is greater than the "total phosphate" present in such an extract he fresh tissue. This is shown by Table I where are given the amounts, at different times, of free and total phosphate present in the trichloroacetic acid extracts of aliquot portions of composite samples of rabbit liver and kidney, which were ground with sand and water and allowed to autolyse in the presence of chloroform. It is probable that this extra phosphate from a non-acid-soluble source is largely derived from lecithin, and it seemed worth while to investigate the enzyme responsible for this hydrolysis.

Table I.

	Kidney		Liver	
	Free phosphate	Total phosphate	Free phosphate	Total phosphate
Days	mg. P in 1 cc. of trichloroacetic acid extract		mg. P in 1 cc. of trichloroacetic acid extract	
0	0·113	0·128	0·211	0·265
1	0·115	—	0·242	—
4	0·131	—	—	0·269
5	0·134	0·134	0·271	—
7	0·141	—	0·289	—
9	0·148	—	0·298	0·306
11	0·149	0·150	0·304	—
13	0·153	—	0·318	—
15	0·155	—	—	—

MacLean [1927] has pointed out that probably in none of the researches on the action of enzymes on lecithin has pure lecithin been used. The obvious impurity of commercial lecithin, must, of course, complicate any results where this material is used as a substrate. One brand of egg-lecithin, which was tried, was only 40 % precipitable by cadmium chloride, and this precipitate, when freed from cadmium and dissolved in ether, gave only a very small precipitate when poured into acetone.

With purified lecithins from different sources, however, there seems to be very little difference in the amount of hydrolysis by the phosphate-splitting enzyme. Table II contains the results of the hydrolysis of three different lecithins by different amounts of enzyme solutions prepared by extracting ground-up beef kidney and small intestine of rabbit with 20 times their weight of distilled water saturated with chloroform. The lecithins used in these experiments were prepared from eggs, from brain, and from Kahlbaum's lecithin, by the method of Levene and Rolf [1927]. Emulsions of the lecithins were made by running 1 volume of 2·5 % alcoholic solution of the lecithin into 10 volumes of hot borate buffer (Palitzsch) at p_H 7·5, with vigorous shaking. This gives a permanent emulsion of lecithin which contains 0·5 mg. of phosphorus in 5 cc. and practically no free phosphate. For the enzymic hydrolysis a series of test-tubes containing 5 cc. of lecithin emulsion and various amounts of the tissue extract were incubated in the presence of a drop of chloroform and toluene for 48 hours at 37·5°. Controls consisted of

5 cc. of borate buffer *plus* the same amounts of extract. Previous work [King and Page, 1930] had shown that there is practically no increase in the free phosphate of a lecithin emulsion itself when incubated at this temperature, so controls of lecithin emulsion *plus* water were omitted. At the end of the 48 hours the hydrolysis was stopped by the addition of 25 % trichloroacetic acid, and the inorganic phosphorus of the filtrate estimated by the Martland and Robison [1924] modification of the Briggs colorimetric procedure. The experiments were done in duplicate and the figures are the average of duplicate determinations.

Table II. *Hydrolysis of different lecithins by varying amounts of tissue extract.*

cc. of extract	Free P extract and buffered lecithin mg.	Free P extract and buffer mg.	Increase in free P mg.
(1) Rabbit intestinal extract and egg-lecithin:			
1	0·208	0·184	0·024
3	0·698	0·545	0·153
5	1·137	0·937	0·200
10	2·280	1·840	0·440
Rabbit intestinal extract and purified Kahlbaum's lecithin:			
1	0·240	0·199	0·041
3	0·714	0·560	0·154
5	1·154	0·948	0·206
10	2·191	1·837	0·354
Rabbit intestinal extract and brain-lecithin:			
1	—	0·161	—
3	0·647	0·546	0·101
5	1·108	0·923	0·185
10	2·243	1·833	0·410
(2) Beef kidney extract and egg-lecithin:			
1	0·176	0·162	0·014
3	0·589	0·462	0·127
5	1·001	0·791	0·210
10	1·951	1·561	0·390
Beef kidney extract and purified Kahlbaum's lecithin:			
1	—	0·162	—
3	0·592	0·462	0·130
5	0·981	0·791	0·190
10	1·869	1·561	0·308

It has been shown by Kay [1926] that different buffer solutions have an effect on the amount of hydrolysis of glycerophosphate by kidney phosphatase. The hydrolysis in presence of borate buffer is always considerably less than that obtained when glycine buffer is used. No such effect as this was noticed in the enzymic hydrolysis of lecithin. The amount of hydrolysis in the presence of borate buffer at p_H 7·5 was practically the same with intestinal and kidney extracts as in the absence of borate, the lecithin emulsion being made up in distilled water and the mixture of lecithin and extract adjusted to p_H 7·5 by the careful addition of acid and alkali. The veronal buffer of Michaelis [1930] is very useful for the range covered by phosphate-hydrolysing enzymes, but appears to have no advantage over the borate mixture of Palitzsch in the

hydrolysis of lecithin, as the extent of hydrolysis appears to be very nearly the same in the presence of either buffer. Thus 2 cc. of rabbit intestinal extract liberated 0·115 mg. of phosphorus from 5 cc. of lecithin emulsion in the presence of veronal buffer and 0·105 mg. in the presence of borate; and 2 cc. of rabbit kidney extract liberated 0·111 mg. phosphorus with the veronal, and 0·103 mg. with the borate buffer during 48 hours' hydrolysis.

The hydrolysis of lecithin in borate buffer at varying times and at different hydrogen ion concentrations is illustrated in Fig. 1. Mixtures of 8 cc. of beef

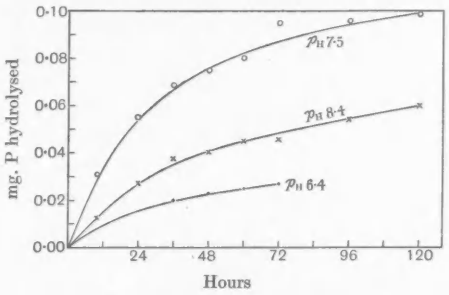

Fig. 1. Hydrolysis of lecithin at different p_H.

kidney extract, 40 cc. of 0·25 % lecithin emulsion (in water) and 40 cc. of borate buffer (of the desired p_H) were kept in the thermostat over a period of several days. 6 cc. portions were removed every 12 hours into 1 cc. of 25 % trichloroacetic acid and the inorganic phosphorus estimated in the filtrate. At the optimal p_H there was a rapid increase in the free phosphate during the first 60 hours, and thereafter a decrease in the rate of hydrolysis until equilibrium seemed to be approached between the fourth and fifth days.

As about 75 % of the hydrolysis had occurred during the first 48 hours, this was adopted as the standard period of time for comparative hydrolysis of lecithin by different tissue extracts. The method used for estimating the amount of the lecithin-hydrolysing enzyme in any tissue is as follows. The sample, freed from adhering tissue, is weighed, ground with sand and transferred with 20 times its weight of water saturated with chloroform to an Erlenmeyer flask. It is allowed to stand at room temperature, with occasional shaking, for 48 hours, when the mixture is filtered through cotton wool, adjusted to p_H 7·5 and preserved with a little toluene in the refrigerator. Longer periods of extraction, frequent grinding of the mixture or repeated extraction with smaller quantities of water do not appear to increase appreciably the amount of enzyme extracted. In the case of kidneys and similar tissues, the whole organ is taken, or it is minced up finely with scissors and an aliquot portion used for grinding and extraction. Intestines are freed from fat and extraneous tissue, split open lengthwise and gently washed out with a little water before mincing. Bones are freed as far as possible from muscle,

split open and freed from marrow before weighing. (Owing to the low "lecithinase" activity of bone, only 10 times the weight of water is used to make the extract instead of 20 times as in the case of other tissues.) To test the enzymic potency of an extract, two test-tubes containing 5 cc. of Palitzsch's borate buffer solution at p_H 7·5, 5 cc. of the 0·25 % lecithin emulsion, and 2 cc. of the tissue extract are incubated at 37·5° in the presence of a drop of chloroform and toluene. Controls consisting of buffer and extract and buffer and lecithin are prepared and incubated in the same way. The amount of free phosphate in the buffer and lecithin tubes is usually very small and may be neglected. At the end of the 48 hours the hydrolysis is stopped by the addition of 3 cc. of 20 % trichloroacetic acid, and the inorganic phosphorus is estimated in the filtrate. The amount of phosphate liberated is taken as a measure of the enzymic activity of the extract and hence of the tissue.

The hydrolysis of lecithin to yield free phosphate probably occurs in two stages, the first of which should yield either a glyceridephosphate or choline phosphate, while the second would involve the cleavage of either or both of these compounds to give free phosphate. Since the glyceridephosphate would probably, and choline phosphate almost certainly, be more soluble than lecithin in trichloroacetic acid solution, it was considered worth while to estimate the total as well as the inorganic phosphorus in the trichloroacetic acid filtrates. In general, the increase in total acid-soluble phosphorus of the tubes containing lecithin and extract over that of the controls ran parallel with, and was slightly greater than, the increase in the inorganic phosphorus.

While phosphatase has an optimum p_H of 8·9 for its action on glycerophosphoric acid, quite different from that of lecithinase, it is conceivable that in some way it may be concerned in the liberation of inorganic phosphorus from lecithin. It was therefore interesting to determine whether the phosphatase activity of the extracts ran parallel with the phosphate-hydrolysing enzyme for lecithin. Such did not appear to be the case. The extracts of bone, which generally showed a high phosphatase activity, were usually without any appreciable action on lecithin. The intestinal and kidney extracts, on the other hand, had a high enzymic activity for both glycerophosphate and for lecithin, but there was considerable variation in the values obtained and no relation could be seen between the phosphatase and lecithinase activities. The method used for determining the phosphatase activity of the extracts consisted in placing 5 cc. of 0·3 % sodium β-glycerophosphate, 5 cc. of glycine buffer and 0·5 cc. of extract (all of p_H 8·9) in each of four test-tubes. 2 cc. of 25 % trichloroacetic acid were added immediately to two of the tubes and the other two were kept at 37·5° in the presence of toluene for exactly 2 hours, and 2 cc. of trichloroacetic acid then added. The increase in free phosphate was taken as the measure of phosphatase activity.

Table III contains the results of a considerable number of determinations of the lecithinase and phosphatase activity of mammalian and avian tissues.

Table III. *Hydrolysis of lecithin and of glycerophosphate by tissue extracts.*

Free P hydrolysed from lecithin mg.	Total P hydrolysed from lecithin (acid-soluble) mg.	Free P hydrolysed from glycero-phosphate mg.
Pig kidney		
0·030	—	0·223
0·020	—	—
Beef kidney		
0·060	—	—
0·031	—	0·179
0·084	—	—
Beef suprarenal		
0·001	—	—
0·012	—	—
Dog kidney		
0·024	—	0·151
0·049	—	0·174
Dog intestine		
0·024	—	0·223
Human baby (acute intestinal diarrhoea)		
Large intestine		
0·006	0·006	0·056
Small intestine		
0·012	0·012	0·069
Human baby (stillborn)		
Small intestine		
0·012	—	0·176
Kidneys		
0·013	—	0·003
Rabbit liver		
0·011	—	0·058
0·022	0·059	0·104
0·006	0·006	0·048
Rabbit suprarenals		
0·008	0·008	0·006
0·008	0·032	0·072
0·011	0·020	0·012
0·003	0·004	0·018
Rabbit pancreas		
0·005	0·049	0·001
0·009	0·059	0·008
0·006	0·006	0·003
Rabbit spleen		
0·015	0·018	0·097
0·021	0·022	—
0·034	0·042	—
0·029	0·039	0·027
Rabbit testes		
0·008	0·011	0·316
0·023	0·024	0·336
Rabbit ovaries		
0·006	0·009	0·101
0·010	0·021	0·059
Rabbit brain		
0·006	0·012	0·021
0·011	0·043	0·022
0·023	0·048	0·033
0·008	0·010	0·020

Free P hydrolysed from lecithin mg.	Total P hydrolysed from lecithin (acid-soluble) mg.	Free P hydrolysed from glycero-phosphate mg.
Rabbit lung		
0·009	0·006	0·065
0·012	0·036	0·105
0·005	0·015	0·074
Rabbit aorta		
0·000	0·010	0·000
0·000	0·009	0·000
Rabbit skeletal muscle		
0·003	0·013	0·005
0·001	0·029	—
0·000	0·007	0·011
0·002	0·005	0·002
Rabbit heart		
0·003	0·024	0·008
0·002	0·002	0·009
0·002	0·013	0·010
0·005	0·006	0·013
Rabbit large intestine		
0·009	0·010	0·014
0·017	0·150	0·024
0·005	0·011	0·019
0·006	0·005	0·015
Rabbit small intestine		
0·024	—	0·075
0·068	—	0·104
0·057	0·088	0·268
0·010	0·010	0·064
0·020	0·064	0·074
0·046	0·227	0·236
0·027	—	0·379
0·020	—	0·040
0·030	0·086	0·219
0·117	—	0·255
0·137	—	0·114
Rabbit kidney		
0·006	—	0·251
0·042	0·063	0·214
0·029	0·036	0·314
0·009	0·030	0·172
0·035	—	0·252
0·024	—	0·112
0·049	0·062	0·224
0·098	—	0·227
0·014	0·060	0·179
Rabbit bone		
0·021	—	0·134
0·010	—	0·231
0·024	0·105	0·307
0·007	0·007	0·286
0·001	0·013	0·309
0·012	—	0·174
0·024	—	0·145
0·009	—	0·112
0·003 (old)	0·022	0·052
0·029 (young)	0·073	0·536
0·076 (rickets)	0·252	0·267
Chicken liver		
0·031	0·038	0·117
0·021	0·032	0·105
0·021	0·016	0·088
0·005	0·016	0 028

Table III (*contd.*).

Free P hydrolysed from lecithin mg.	Total P hydrolysed from lecithin (acid-soluble) mg.	Free P hydrolysed from glycero-phosphate mg.
Chicken gizzard		
0·004	0·007	0·004
0·001	0·017	0·004
0·000	—	0·007
0·008	0·017	0·009
Chicken pancreas		
0·006	0·019	0·012
0·002	0·031	0·014
0·041	0·083	0·012
0·022	0·021	0·005
Chicken spleen		
0·010	0·054	0·018
0·020	0·069	0·012
0·001	0·020	0·009
Chicken proventriculus		
0·013	0·031	0·015
0·017	0·038	0·029
0·002	0·037	0·010
Chicken testes		
0·040	0·061	0·096
0·012	0·024	0·047
0·008	0·010	0·008
0·013	0·017	0·055
0·007	0·016	0·039
0·011	0·060	0·051
0·016	0·059	0·027
Chicken brain		
0·010	0·018	0·036
0·008	0·027	0·041
0·006	0·011	0·024
0·015	0·015	0·016
Chicken lung		
0·006	0·017	0·023
0·001	0·007	0·011
0·002	0·002	0·011
0·011	0·017	0·054
Chicken blood vessels		
0·014	0·020	0·037
0·001	0·004	0·005
0·009	0·014	0·039
Chicken skeletal muscle		
0·005	0·007	0·008
0·002	0·017	0·004
—	0·008	0·002
0·007	0·007	0·005
Chicken cardiac muscle		
0·001	0·002	0·018
0·004	0·017	0·008
0·008	0·016	0·013
Chicken large intestine		
0·031	0·065	0·062
0·005	0·031	0·021
0·012	0·068	0·017
0·021	0·033	0·014
Chicken small intestine		
0·023	0·039	0·027
0·015	0·091	0·030
0·019	0·189	0·035
0·046	0·056	0·051
0·012	0·125	0·066
0·008	0·082	0·032
0·006	0·110	0·034
0·006	0·076	0·018
0·007	0·036	0·050
Chicken kidney		
0·022	—	0·230
0·017	0·022	0·059
0·031	0·088	0·138
0·038	0·178	0·183
0·053	0·054	0·344
0·016	0·043	0·277
0·012	0·023	0·227
0·032	—	0·256
0·036	0·079	0·260
0·020	0·058	0·254
0·026	0·032	0·196
Chicken bone		
0·020	—	0·331
0·004	—	0·046
0·000	0·005	0·035
0·003	0·035	0·185
0·007	0·010	0·157
0·011	0·024	0·358
0·021	0·024	0·387
0·016	—	0·178
0·028	—	0·076
0·001	0·044	0·085
0·000	0·037	0·110
0·003	0·008	0·132

Inactivation of the enzyme.

When an active tissue extract is kept at body temperature at different hydrogen ion concentrations, a marked destruction of the enzyme occurs at reactions which are not near neutrality. Fig. 2 illustrates the results which were obtained on the inactivation of an extract of rabbit kidney. 10 cc. quantities of the extract were adjusted to different p_H by the careful addition of a few drops of $N/10$ acid or alkali. After being left in the thermostat at 38° for 24 hours, the samples were adjusted to p_H 7·6 by adding more acid or alkali. Water was then added to all of them to bring the volume to 12 cc.

The lecithinase and phosphatase activities were determined on 2 cc. and 0·5 cc. portions, respectively, and the results compared with the activity of the original extract. Between p_H 7·0 and 8·0 there appeared to be very little loss of activity towards either lecithin or glycerophosphate. Above p_H 8, and below p_H 6, however, there was a marked diminution in the activity of the extracts.

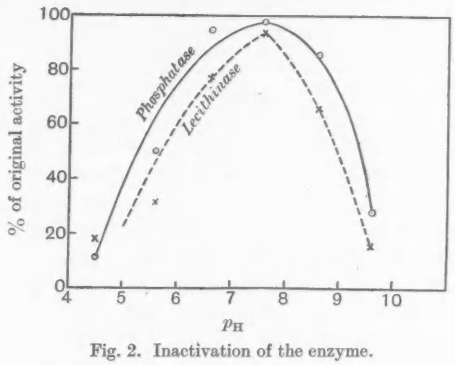

Fig. 2. Inactivation of the enzyme.

Hydrolysis of lecithin at different temperatures.

The optimum temperature for the hydrolysis of lecithin appears to be that of the body. Incubation of hydrolysing mixtures at lower or higher temperatures than 38° results in a smaller increase in the free and total acid-

Table IV. *Hydrolysis of lecithin at different temperatures.*

	Days	Free P mg.	Increase in free P mg.	Total acid-soluble P mg.	Increase in total acid-soluble P mg.
(1) Temperature 11°	0	0·220	—	0·244	—
	1	0·221	0·001	0·245	0·001
	2	0·223	0·003	0·246	0·012
	3	0·224	0·004	0·251	0·007
	4	0·227	0·007	0·254	0·010
	5	0·231	0·011	0·266	0·022
(2) Temperature 23°	0	0·220	—	0·244	—
	1	0·224	0·004	0·253	0·009
	2	0·235	0·015	0·275	0·031
	3	0·245	0·025	0·278	0·034
	4	0·254	0·034	0·285	0·041
	5	0·261	0·041	0·302	0·058
(3) Temperature 38°	0	0·220	—	0·244	—
	1	0·232	0·012	0·262	0·018
	2	0·252	0·032	0·272	0·028
	3	0·261	0·041	0·298	0·054
	4	0·276	0·056	0·296	0·052
	5	0·288	0·068	0·314	0·070
(4) Temperature 57°	0	0·220	—	0·244	—
	1	0·221	0·001	0·261	0·017
	2	0·226	0·006	0·261	0·017
	3	0·231	0·011	0·282	0·038
	4	0·240	0·020	0·278	0·034
	5	0·244	0·024	—	—

soluble phosphorus. Two test-tubes, each containing 20 cc. of buffer (p_H 7·5), 20 cc. of lecithin emulsion and 10 cc. of kidney extract, were kept in thermostats at different temperatures for a period of 5 days, 8 cc. being withdrawn each day into 2 cc. of 25 % trichloroacetic acid for the determination of free and total phosphorus (Table IV).

The effect of hydrolysis products on the rate of hydrolysis.

If the velocity constants for a unimolecular reaction,

$$k = \frac{1}{t} \log \frac{a}{a-x},$$

are calculated from the data of hydrolysis experiments, it is seen that there is a very marked difference in the behaviour of the hydrolysing mixtures according to the enzyme preparation used. For some kidney preparations the values for k are fairly constant over a period of several days. For others, and especially for intestinal preparations, the reaction velocities show a marked and steady decline and soon reach values which are much smaller than those for the original rates of hydrolysis. The data for four experiments which were carried out to test this point are contained in Table V.

Table V. *Rate of hydrolysis of lecithin.*

Time hrs.	Free P hydrolysed mg.	k	Time hrs.	Free P hydrolysed mg.	k	Total acid-soluble P hydrolysed mg.	k
Rabbit kidney extracts:							
24	0·021	0·000778	8	0·017	0·00186	0·037	0·00418
48	0·034	0·000648	24	0·058	0·00223	0·093	0·00372
72	0·062	0·000803	32	0·073	0·00214	0·109	0·00333
96	0·083	0·000824	48	0·103	0·00209	0·143	0·00304
120	0·091	0·000731	59	0·122	0·00206	0·170	0·00305
144	0·106	0·000722	72	0·127	0·00176	0·173	0·00255
168	0·120	0·000714	94	0·136	0·00147	0·170	0·00192
192	0·120	0·000623	Total lecithin-P 0·500 mg.				
216	0·127	0·000592					
240	0·134	0·000567					
Total lecithin-P 0·500 mg.							
Rabbit intestinal extracts:							
24	0·159	0·00693	24	0·012	0·000566	0·031	0·001461
48	0·171	0·00379	48	0·013	0·000302	0·032	0·000759
72	0·191	0·00290	72	0·012	0·000189	0·042	0·000673
96	0·199	0·00229	96	0·015	0·000176	0·051	0·000622
120	0·228	0·00220	120	0·023	0·000214	—	—
144	0·231	0·00187	144	0·024	0·000189	0·069	0·000572
168	0·232	0·00161	168	0·029	0·000196	0·077	0·000553
192	0·233	0·00142	192	0·031	0·000184	0·077	0·000484
216	0·239	0·00130	216	0·033	0·000174	—	—
240	0·244	0·00121	Total lecithin-P 0·400 mg.				
Total lecithin-P 0·500 mg.							
Enzyme solutions dialysed 48 hours against running water.			Enzyme solutions electrodialysed against tap-water till nearly free of inorganic phosphate.				

It would seem that this decline in the values of k is more probably due to the slow destruction of the enzyme in the hydrolysing mixture than to the

accumulation of the products of hydrolysis. The increases of inorganic phosphate in the experiments cited are not sufficiently different in amount to account, on the basis of inhibition due to accumulation of a product of hydrolysis, for the large variations in the values for the velocity constants.

Several attempts were made to ascertain the effect on the rate of hydrolysis of added inorganic phosphate, but such small differences as were observed in the amounts hydrolysed were irregular and could not be attributed to the extra phosphate present.

Since the cleavage of inorganic phosphorus from lecithin also involves the removal of choline, it was of interest to determine what influence, if any, choline had on the amount of hydrolysis as estimated by the liberation of phosphate. Table VI contains the results of an experiment in which choline, in varying amounts, was added to digesting mixtures of lecithin and rabbit kidney extract. The addition of the choline caused no diminution in the amount of phosphorus liberated, and the variations in the values for k between the solutions of different choline content are no greater than the variations in the values for the individual solutions.

Table VI. *Effect of choline on the hydrolysis of lecithin.*

	No choline				0·005 *M* choline			
Time hrs.	Free P hydrolysed mg.	k	Total acid-soluble P hydrolysed mg.	k	Free P hydrolysed mg.	k	Total acid-soluble P hydrolysed mg.	k
14	0·020	0·001286	0·031	0·001995	0·020	0·001286	0·030	0·001939
28	0·028	0·000899	0·042	0·001331	0·030	0·000964	0·041	0·001336
38	0·036	0·000863	0·056	0·001362	0·040	0·000960	0·056	0·001369
49	0·055	0·001035	0·054	0·001021	0·055	0·001035	0·057	0·001081
62	0·060	0·000901	0·061	0·000917	0·061	0·000918	0·068	0·001031
75	0·066	0·000822	0·072	0·000906	0·071	0·000892	0·075	0·000948
86	0·067	0·000730	0·084	0·000933	0·074	0·000814	0·082	0·000907
96	0·078	0·000771	0·074	0·000729	0·081	0·000802	0·083	0·000824
110	0·095	0·000836	0·085	0·000737	—	—	—	—
	0·01 *M* choline				0·04 *M* choline			
14	0·023	0·001499	0·022	0·001421	—	—	0·022	0·001421
28	0·033	0·001071	0·039	0·001268	0·028	0·000899	0·030	0·000968
38	0·041	0·000984	0·054	0·001314	0·040	0·000960	0·048	0·001157
49	0·054	0·001017	0·052	0·000980	0·052	0·000980	—	—
62	0·064	0·000967	0·064	0·000967	0·060	0·000914	—	—
76	0·072	0·000904	—	—	0·067	0·000840	—	—
86	0·076	0·000837	0·078	0·000861	0·072	0·000790	—	—
96	—	—	—	—	0·075	0·000739	0·077	0·000761
110	0·094	0·000827	—	—	—	—	—	—

Total lecithin-P 0·500 mg.

Enzyme solution electrodialysed against tap-water.

The presence of glycerol in the hydrolysing mixtures had no effect in concentrations up to 0·4 *M*, the highest investigated. An experiment similar to the foregoing was carried out with lecithin, kidney extract and varying amounts of added glycerol. A sample equivalent to 0·500 mg. of lecithin-P

was removed from each flask at various periods and the free and total P estimated in the trichloroacetic acid filtrates (Table VII).

Table VII. *Effect of glycerol on the hydrolysis of lecithin.*

	No glycerol		0·01 *M* glycerol		0·1 *M* glycerol		0·4 *M* glycerol	
Time hrs.	Free P hydrolysed mg.	Total acid-soluble P hydrolysed mg.	Free P hydrolysed mg.	Total acid-soluble P hydrolysed mg.	Free P hydrolysed mg.	Total acid-soluble P hydrolysed mg.	Free P hydrolysed mg.	Total acid soluble P hydrolysed mg.
13	0·007	0·019	0·007	0·011	0·003	0·003	0·009	0·010
24	0·019	0·020	0·020	0·027	0·015	0·013	0·014	0·014
35	0·017	0·031	0·018	0·026	0·016	0·016	0·013	—
48	0·030	0·033	0·032	0·024	0·027	—	0·027	0·031
61	0·030	0·050	0·033	0·048	0·027	0·032	0·031	0·039
80	0·042	0·054	0·042	0·054	0·037	—	0·037	0·040
95	—	0·059	0·046	0·058	0·043	0·051	0·041	0·042
105	0·044	0·059	0·046	0·056	—	—	0·041	0·044
120	0·057	0·060	0·057	0·063	0·045	—	0·059	—

Summary.

1. An enzyme capable of hydrolysing lecithin to liberate inorganic phosphate is present in various animal tissues.

2. The optimal hydrogen ion concentration for the action of this enzyme is at about p_H 7·5, while that of phosphatase is at p_H 8·9. For this reason, and because the distribution of "lecithinase" does not correspond with that of phosphatase, they are thought not to be the same enzyme.

3. The relative "lecithinase" activity of different tissues is as follows: (in decreasing order)—kidney, small intestine, spleen, liver, testes, pancreas, large intestine, brain, ovaries, bone, suprarenals, lung, blood vessels, cardiac muscle, skeletal muscle.

The enzyme is fairly stable at a neutral reaction but is rapidly destroyed when kept in an acid or alkaline medium at 38°.

4. Lecithin appears to be hydrolysed most rapidly by the enzyme at body temperature.

5. The addition of inorganic phosphate and choline (products of hydrolysis) and of glycerol to hydrolysing mixtures of lecithin and the enzyme does not appear to affect appreciably either the rate or amount of hydrolysis.

The technical work and colorimetric analyses in connection with this investigation were done by Miss Margaret Shaw.

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XCI. THE NATURE OF THE "ETHER REACTION" OF URINE.

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(*Received April 22nd, 1931.*)

Over forty years ago Plosz [1890] showed that when urine is strongly acidified with acetic acid and shaken with ether, chloroform or amyl alcohol, the mixture separates into two layers and on the boundary between the two a precipitate of protein is formed. He remarks: "The reaction is given by all urines, since all urines contain protein. The delicacy of the reaction is thereby shown[1]. The protein so obtained is not a homogeneous substance since only a portion of it is soluble in acetic acid. Another portion is soluble in water, alkalis, and strongly concentrated solutions of common salt. The fraction insoluble in acetic acid dissolves, after being washed with acetic acid, in dilute alkali, and is again precipitated on addition of acetic acid. It behaves like mucin. Normal urine always contains protein and a body behaving in its reactions like mucin."

Two years later Smith [1892], who evidently had not seen Plosz's paper, described a reaction of urine with ether in which the acidified urine was shaken with ether, and a thick scum was formed as the layers separated. He stated that the scum was most abundant in the urine of those who had a good appetite and good digestion and was usually absent after long fasting or when the diet was greatly restricted.

Oriel and Barber [1930] have found that when acidified urine is shaken with one-fifth of its volume of ether the mixture separates into two layers with a scum between, which varies in extent and consistency according to the health of the person from whom the urine is derived. When the aqueous layer is discarded and alcohol is added to the ether and scum a precipitate is obtained which gives the biuret, Millon, Adamkiewicz and Molisch reactions. They state that the nitrogen content of the precipitate is also variable and in allergic conditions may rise from a few mg. to as much as 300 mg. daily with the onset of an attack, subsiding to its original value as the attack passes. The chief interest of Oriel and Barber's observations, however, lies in their discovery that the precipitate obtained from urine passed during an attack, contains some of the antigen responsible for the attack as shown by skin reactions and by reproduction of the attack when a solution of the precipitate is injected intradermally into the patient from whom it has been derived.

[1] That is, because the boiling test is negative in normal urines.

It appeared to be desirable to investigate the nature of this ether-alcohol precipitate more closely.

Isolation of the crude material.

The material was obtained by treating 500 cc. of urine with 5 cc. of 25 % sulphuric acid, adding 100 cc. of ether, shaking the mixture vigorously in a stoppered separating funnel for a minute and allowing the ether to separate for 15 minutes. The aqueous layer was then discarded and 100 cc. of 93 % alcohol were added to the ether and the scum. The precipitate was allowed to settle and was separated by centrifuging, washed with alcohol and with ether and dried in a desiccator or at 100° as required.

The material could also be obtained by adding to the urine 3 vols. of 93 % alcohol, and collecting the precipitate. The proportion of salts present was, however, much higher in this case.

The quantity of material obtained by means of the ether reaction as described above appeared to be higher in the case of urines from allergic patients than in the case of urines from apparently normal subjects. Thus an asthmatic in an attack yielded 1·067 g. per litre containing 0·047 g. N, whereas men employed on indoor work and apparently normal yielded 0·165 g. per litre, containing 0·004 g. N. Only a few allergic urines, however, have been examined quantitatively in this way, because Oriel and Barber have provided abundant data and also because the values so obtained are liable to certain errors. As will be shown the material consists largely of salts precipitated by the alcohol from the urine entangled in the ether, and some nitrogenous substances are similarly precipitated or adsorbed, which have no direct relation to the ether test. Further, only after being shaken several times with fresh quantities of ether does the urine cease to give the typical honeycomb layer. The vigour of the shaking also materially affects the degree of emulsification, and so the quantity of material obtained by a single treatment.

Ash content of the crude material.

The ash content of the precipitate (determined by ignition at a moderate temperature, moistening the ash with dilute sulphuric acid and then strong ignition) varied from 70 to 87 % of the material which had been dried at 100°. It consisted, as might be expected, of sodium sulphate with about 6 % of calcium sulphate and sometimes a little phosphate.

Fraction of the crude material soluble in cold water.

The greater part of the crude material was soluble in water and the solution obtained, using only a small excess of cold water above that required to dissolve the salts, gave only a moderate ether reaction. The p_H of this solution was about 3. The solution did not give the protein reactions distinctly but gave Molisch's reaction which was probably due to a trace of glucose in this

case. Evidently the material responsible for the ether reaction is sparingly soluble in concentrated salt solution at p_H 3.

Of the crude dry material from urine passed during a severe attack of asthma 89 % was soluble under these conditions. The crude material had 4·5 % N, of which 3·2 % was soluble, including 0·4 % of ammonia-N (these figures being all percentages of the crude material). The soluble portion contained practically all the inorganic matter and some organic matter. No appreciable precipitate was obtained on saturating the solution with ammonium sulphate and boiling. As the substance responsible for the ether reaction was almost entirely in the insoluble fraction the soluble material was not examined further.

The protein fraction.

The residue obtained on washing the crude material free from salts with cold water will be referred to as the "protein fraction" because it consisted almost entirely of protein or related substance. It was soluble to some extent in cold water, even after acidification, and could not therefore readily be obtained free from salts by shaking the ether with acidified water after removal of the urine. In its moist finely divided state it dissolved instantly in very dilute alkali to give an almost clear, slightly brown solution. When dried at 100° for some time it became insoluble in dilute ammonia. Addition of acetic acid to the filtered alkaline solution produced slight opalescence but no precipitate in the cold. On boiling the acidified solution a little coagulated protein was precipitated. When this had been removed the filtrate, although giving all the colour reactions of proteins, did not give a precipitate with potassium ferrocyanide. This applies both to material from asthmatic and that from non-asthmatic urine.

Half saturation of the acidified solution (freed from coagulable protein) with ammonium sulphate gave only a trace of precipitate in the cold even after 12 hours, but, on boiling, a flocculent precipitate was obtained and the filtrate from this, both in the case of asthmatic and normal urine was found to be free from protein. The precipitate was soluble in hot water and did not come down again on cooling.

When the acetic acid solution (freed from coagulable protein) was saturated completely with ammonium sulphate a precipitate was obtained in the cold. This was removed by centrifuging, dissolved in water, dialysed until free from sulphate and reprecipitated by addition of 3 vols. of alcohol. The precipitate was separated, washed with alcohol and ether and dried at 100°. It formed slightly pigmented transparent scales. It was acid to litmus and was readily soluble in water at p_H 8. It was precipitated by Esbach's reagent, but not by ferrocyanide. It contained no phosphorus, and had 12·5 % N (micro-Kjeldahl) on an ash-free basis. By an adaptation of Weiss's modification of Millon's reaction the tyrosine content of material from asthmatic urine was estimated to be about 2 %, and the tryptophan by the May and Rose colorimetric method as modified by Boyd [1929] was found to be 0·5 %. The

glyoxylic reaction was definite but faint. Cystine was present as shown by the lead acetate test. Molisch's test was positive. The low nitrogen content, together with the low tryptophan content, points to the presence of a large prosthetic group. This is confirmed by the behaviour of the material on heating with Fehling's solution, slow but distinct reduction taking place. A control test, using purified coagulated fish-muscle protein, showed no reduction under the same circumstances. It appears probable therefore that carbohydrate is present in combination with a protein.

The only difference observed hitherto between the material isolated from normal and that from asthmatic urines is one of quantity.

Determination of the protein nitrogen of urine.

If a sediment is present in the urine the sample is first made alkaline to dissolve uric acid and protein. 25 cc. of the filtered urine are acidified with acetic acid and 75 cc. of 93 % alcohol are added. After 12 hours the precipitate is separated on the centrifuge, dissolved in water, and the solution is dialysed in a thin collodion membrane for a week in presence of toluene, the water being frequently changed. The contents of the membrane are then transferred to an evaporating basin, evaporated to 5 cc. and transferred to a hard glass boiling-tube, and the nitrogen content is determined. Almost identical results may be obtained by direct dialysis of the urine without alcohol precipitation, but by the above method the urea, hippuric acid and other substances are eliminated first.

Protein nitrogen of normal and pathological urines.

A few normal and pathological urines have been examined by the above method, and the results are given in Table I. Further work is being carried out in this direction, but from the present results it is evident that there may be a normal urinary protein content even in the cachexia of cancer. The constancy of an increased protein excretion in allergic conditions in particular is being investigated.

Table I. *Protein* (i.e. *non-dialysable*) *nitrogen of some normal and pathological urines.*

Urine	Non-dialysable nitrogen (g. per litre)
1. Normal (apparently)	0·014
2. Normal (apparently)	0·024
3. Cancer (extreme cachexia)	0·011
4. Cancer	0·016
5. Cerebral tumour	0·009
6. Besnier Prurigo	0·053
7. Asthma (in attack)	0·030
8. Cow (healthy)	0·020

Discussion of the nature of the protein.

Considerable confusion exists as regards protein substances in urine. The term "proteose" has often been employed loosely. From the description of the product isolated from normal and asthmatic urines it appears to be a mixture of heat-coagulable protein and a mucoid. The practical differentiation between mucoids and glucoproteoses is not clear. Phosphorus was not found in the purified material, *i.e.* after precipitation and dialysis, but the crude protein fraction from one asthmatic urine contained 0·3 % of phosphorus. It appears that little or no nucleoprotein is present.

Mörner [1895] made a detailed study of protein substances in normal urine. He found a substance which he called "urinary mucoid" identical with the well-known "nubecula" of cooled urine and obtained by him by allowing the filtered fresh urine to stand in presence of chloroform. Its composition and reactions corresponded closely with those described above for the substance isolated by means of the ether reaction, after removal of irreversibly heat-coagulable protein. He also found that normal urine contains a little serum-albumin, which, on addition of acetic acid to urine and shaking with chloroform, is precipitated chiefly in combination with chondroitinsulphuric acid, but also in combination with glycuronic acid and nucleic acid. The properties of the mucoid and of the loose compound of serum-albumin with chondroitin-sulphuric acid, as found by Mörner, were very similar, but the mucoid was not precipitated on shaking its solution with organic solvents in presence of chondroitinsulphuric acid (which is said to be present in all urines), although it was so precipitated in the absence of that substance. On this his separation of two different mucin-like substances depended. A consideration of the precipitation reactions of these bodies and of the phenomenon of the precipitation of proteins on the interfaces between solvents and water throws doubt on Mörner's conclusion that the mucoid was not precipitated from urine by shaking with solvents.

In order to settle this point 100 cc. of fresh normal urine were acidified with sulphuric acid and shaken repeatedly with fresh quantities of ether until a honeycomb layer was no longer obtained. The aqueous layer was then treated with 3 vols. of 93 % alcohol and after some hours the precipitate was removed on the centrifuge, dissolved in water, and the solution dialysed for a week in presence of toluene, the water being frequently changed. The non-dialysable nitrogen was then determined. The protein nitrogen of the urine was similarly determined omitting the treatment with ether. It was found that whereas the urine contained 0·014 g. of protein nitrogen per litre, the protein nitrogen left after repeated treatment with ether was 0·0026 g. per litre. Mörner repeatedly found mucoid corresponding to about 0·003 g. of nitrogen per litre, but it is probable that the precipitation was by no means complete. Further, it is not to be expected that shaking with ether can effect complete separation of the protein. It seems likely that the 0·0026 g. per litre of residual

non-dialysable nitrogen was due partly at least to a residue of the same kind of protein as that precipitated. These considerations support the view that Mörner was mistaken in supposing that he had effected a separation of two different kinds of mucin-like substances. Both, if present, are precipitated on shaking the acidified urine with solvents.

The irreversibly-coagulable protein (from asthmatic urine) was boiled repeatedly with water faintly acidified with acetic acid, allowed to stand in water at p_H 8 for 15 hours, washed repeatedly with distilled water by decantation, and then with alcohol and ether and dried at 100°. Its nitrogen content, allowing for 1·3 % of ash present, was 14·7 %. The alkaline treatment was intended to remove loosely combined precipitants such as chrondroitinsulphuric acid, mentioned by Mörner. As the nitrogen content of serum-albumin is 15·9 % it is possible that the coagulated material was serum-albumin contaminated with mucoid (which, according to Mörner, is itself a protein precipitant) or with chondroitinsulphuric acid.

It is hoped by means of biological tests to establish the identity or non-identity of the proteins of normal and allergic urines with components of blood-serum and to confirm Oriel and Barber's observations on the specific antigenic properties of the urinary protein of allergic conditions, using purified material.

Conclusion.

The substances in normal and asthmatic urines responsible for the ether reaction and isolated thereby are an irreversibly coagulable protein, possibly identical with serum-albumin, and a mucoid containing 12·5 % of nitrogen, possibly derived from the urinary passages.

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XCII. NOTE ON THE REACTIVATION OF REDUCTASE IN WASHED YEAST PREPARATIONS.

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It was shown by Harden and Norris [1914, 1915] that after being washed with water dried (Lebedev) yeast did not reduce methylene blue; this property however was restored on the addition of boiled washings and also of definite chemical substances such as salicylaldehyde, benzaldehyde and lactate. It was suggested by Lebedev and Griaznov [1912] that a co-reductase was necessary for the action of reductase, in a manner analogous to that of co-zymase; this theory, though later withdrawn by Lebedev [1927] is upheld by Euler and his colleagues [Euler and Nilsson, 1925] who regard the co-reductase as being identical with co-mutase and the co-enzyme of alcoholic fermentation. In considering the evidence for this view the possibility must be borne in mind that boiled yeast extract may restore the activity of the reductase only by reason of hydrogen donators contained therein rather than by the presence of a true co-enzyme. Euler and Nilsson [1927] however showed that neither a co-enzyme preparation which had been considerably purified nor a hexose-diphosphate diminished the time required for the reduction of methylene blue by washed dried yeast when added separately, whereas when both were present together decoloration took place rapidly. If however the addition of co-enzyme together with hexosediphosphate, aldehyde or other substance induces alcoholic fermentation in a washed preparation, the chemical changes involved may provide an easily oxidisable substance which will allow the reduction of methylene blue to proceed, without any necessity for postulating a co-reductase. Consequently, it is unjustifiable to draw any conclusions as to the necessity for a co-reductase from experiments on the reactivation of reductase in washed yeast by the addition of any substance, if the possibility exists that such a reactivation is a secondary effect of alcoholic fermentation induced by the substance.

The earlier experiments of Harden and Norris were made at a time when failure to ferment sugar in presence of inorganic phosphate was accepted as evidence of the complete absence of co-enzyme from the washed yeast preparation. Meyerhof [1918] showed that the presence of a small amount of hexosediphosphate rendered this test more delicate, since its fermentation required a smaller concentration of co-enzyme. Later Kluyver and Struyk

[1927, 1928] showed that the reactivation of apozymase by acetaldehyde described by Harden [1917] did not take place in maceration residue after adequate washing; while Stheeman [1930] has made it clear that four distinct stages of inactivation of the fermenting complex of zymin can be attained by various degrees of washing. In the first stage the washed zymin is reactivated by a hydrogen acceptor, such as methylene blue or acetaldehyde; in the second by hexosediphosphate; in the third by hexosediphosphate and a hydrogen acceptor, and in the last by hexosediphosphate and co-enzyme. It has been confirmed in this laboratory that the adequate criterion for absence of co-enzyme in a washed preparation is a failure to ferment in presence of hexosediphosphate, and that the reactivation by acetaldehyde previously described [Harden, 1917] does not take place in the complete absence of co-enzyme.

It is obvious that a preparation in any of the first three stages of inactivation of zymase may be reactivated by aldehydes and hexosediphosphate with regard to reductase, if the fermentation so induced does in fact provide a suitable hydrogen acceptor. It seemed probable that the results obtained by Harden and Norris [1914] might require revision in the light of the later evidence as to the stages of inactivation by washing. It was therefore decided to repeat their experiments on the reactivation of reductase by chemical substances, using a washed preparation whose state of inactivation was checked by simultaneous fermentation experiments. Since it was impossible to use the same type of yeast as before, dried baker's yeast was chiefly used, as this is easily freed from co-enzyme. The results given below were obtained from various washed preparations of dried yeast.

Preparation 1. Dried baker's yeast washed three times by centrifuging with 8 parts of water, once on the filter with 5 parts, and dried with acetone and ether.

Preparation 2. Same dried yeast, washed four times with 8 parts of water by centrifuging, once on the filter and suspended in water (5 parts).

Preparation 3. Same dried yeast, washed five times with 8 parts of water, once on the filter with 5 parts and suspended in water (5 parts).

Preparation 4. Dried Copenhagen yeast, washed five times with 8 parts of water by centrifuging, once on the filter and suspended in water (5 parts).

Fermentation experiments. 0·2 g. of the washed preparation, 0·5 cc. of 20 % fructose and 0·1 cc. 0·6 *M* K_2HPO_4 were placed in each of three micro-fermentation flasks to which were added:

Flask (*a*) 0·5 cc. 0·1 *M* hexosediphosphate;
(*b*) 1·0 cc. yeast extract (prepared from ale yeast zymin);
(*c*) 0·5 cc. hexosediphosphate + 1·0 cc. yeast extract;

together with water to make a total volume of 2·5 cc. The flasks were placed in a thermostat at 30°, the CO_2 evolved being measured (Table I).

Reductase experiments. The reducing action of the preparations towards

methylene blue was then tested in the presence of various substances. A measured quantity of the washed suspension with 0·1 cc. of 1/1000 methylene blue and 0·1 cc. toluene was incubated at 26° in a total volume of 4 cc. with the addition of the desired substance. After constant observation for some hours, the mixtures were allowed to stand overnight, the minimum reduction time being given for such as were decolorised in the morning (Table II).

Table I. *Fermentation by washed yeast preparations. cc. CO_2.*

	Washed preparation							
	1		2		3		4	
Time in hrs.	2	20	2	20	2	20	2	20
Flask (*a*)	0	>5	0	>5	0	0·15	0	0·25
(*b*)	0·06	>5	0	>5	0	0	0·06	1·45
(*c*)	1·49	>5	3·16	>5	2·6	>5	0·24	2·20

Table II. *Reduction time of methylene blue by washed preparations.*

	Preparation			
Additions	1	2	3	4
(*a*) —	>5 hrs.	>4 hrs.	>4 hrs.	>3 hrs.
(*b*) 0·5 cc. hexosediphosphate	>5 hrs.	3¼ hrs.	3 hrs.	>3 hrs.
(*c*) 1 cc. yeast extract	>5 hrs.	15 min.	—	5 min.
(*d*) 1 cc. yeast extract + 0·5 cc. hexosediphosphate	20 min.	15 min.	—	5 min.
(*e*) 0·01 cc. salicylaldehyde	>5 hrs.	>4 hrs.	>4 hrs.	>3 hrs.
(*f*) 0·01 cc. benzaldehyde	>5 hrs.	>4 hrs.	>4 hrs.	>3 hrs.
(*g*) 0·1 cc. 0·01 *M* lactate	1 hr.	30 min.	20 min.	15 min.
(*h*) 0·1 cc. lactate + 0·5 cc. hexosediphosphate	45 min.	—	20 min.	—
(*i*) 0·1 cc. 0·01 *M* succinate	—	3 hrs.	—	—

Another sample of washed yeast did not reduce methylene blue after 20 hours alone, but did so in 30 minutes with yeast extract, 1¼ hours with lactate and 2¼ hours with methylglyoxal.

The washed yeast preparations varied somewhat in their properties according to the manner of preparation. Thus preparation 1, which had been treated with acetone, required both yeast extract and hexosediphosphate for a rapid reduction, while preparation 2 reduced rapidly with yeast extract alone, although there was no significant difference in their fermenting power. The Copenhagen yeast reduced quickly with yeast extract, but fermented very poorly. The preparations were all consistent in that in the absence of yeast extract and hexosediphosphate, neither fermentation nor reduction took place during the period of constant observation (2–6 hours), though in the majority of cases both fermentation and reduction took place overnight. In all cases the addition of lactate caused a reduction of methylene blue in a time comparable with that taken by an addition of yeast extract + hexosediphosphate. With preparation 3, for instance, where no fermentation occurred in absence of co-enzyme, lactate effected reduction in 20 minutes, and with preparation 5, which did not reduce methylene blue alone, in 1¼ hours. Succinate and methylglyoxal were also capable of acting as hydrogen donators,

though less rapidly than lactate but salicylaldehyde and benzaldehyde were ineffective.

During the reduction of methylene blue by washed dried yeast in presence of lactate, the lactate is decomposed with evolution of CO_2, presumably according to the equations

$$CH_3.CHOH.COOH + Mb \rightarrow MbH_2 + CH_3.CO.COOH,$$
$$CH_3.CO.COOH \rightarrow CH_3.CHO + CO_2,$$

the pyruvic acid being decomposed by the carboxylase of the yeast. The acetaldehyde produced might conceivably also have acted as an acceptor for the dehydrogenation of lactate. That this was not the case was shown in the following experiments.

0·2 g. washed yeast + 0·25 cc. K_2HPO_4 were placed in each of three microfermentation flasks to which were added

1. 1 cc. 1 % lactate + 0·01 g. methylene blue + 1·25 cc. H_2O;
2. 1 cc. lactate + 0·03 g. methylene blue + 1·25 cc. H_2O;
3. 1 cc. lactate + 0·1 cc. 10 % acetaldehyde + 1·24 cc. H_2O.

The flasks were placed in a thermostat at 30° and the CO_2 measured. Flask 1 became decolorised in 45 minutes, giving 0·73 cc. CO_2; flask 2 in 80 minutes, giving 1·82 cc. CO_2, the theoretical amounts for these quantities of methylene blue being 0·8 and 2·4 cc. respectively. No evolution of CO_2 took place in flask 3. Rather less than the equivalent amount of CO_2 was evolved for the methylene blue reduced, showing that the acetaldehyde produced in the reaction or added independently did not act as a hydrogen acceptor for lactate. Moreover, the addition of pyruvic acid, which might liberate acetaldehyde in a more reactive state, was also ineffective in inducing a fermentation of lactate.

Summary.

The addition of lactate, succinate or methylglyoxal restored the power of reducing methylene blue to yeast preparations washed to a point at which no fermentation of hexosediphosphate occurred. From these results it is concluded that a co-enzyme is probably not required for the action of the reductase in yeast preparations.

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XCIII. FERMENTATION BY YEAST PREPARATIONS.

I. THE EFFECT OF MONO-IODOACETATE ON THE FERMENTATION OF HEXOSEDIPHOSPHATE.

II. THE ACTION OF ARSENATE ON THE INDUCTION PERIOD OF ZYMIN.

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(*Received April 20th, 1931.*)

I. The effect of mono-iodoacetate on the fermentation of hexosediphosphate.

It has been shown [Macfarlane, 1930] that a yeast hexosephosphatase incapable of esterifying and fermenting sugar can be obtained, whose action in liberating inorganic phosphate from hexosediphosphate is accelerated by arsenate only in the presence of the co-enzyme of alcoholic fermentation and with an equivalent production of CO_2. Fluoride, which inhibits the hydrolysis either in presence or absence of arsenate only by about 10 %, decreases the fermentation of hexosediphosphate in presence of arsenate by 80 %.

According to Lohmann [1930] both muscle extract and yeast maceration juice in the presence of fluoride convert glycogen or starch or added hexosediphosphate into a diphosphoric ester much more resistant to acid hydrolysis than the Harden and Young ester. Lipmann [1930] and Lundsgaard [1930, 1] have shown that the poisoning of muscle by sodium fluoride and by mono-iodoacetate produces similar effects, *i.e.* the muscle can contract without formation of lactic acid but with the formation of a hexosediphosphoric ester, probably of the same type as Lohmann's ester. Moreover mono-iodoacetate prevents the esterification of sugar and phosphate by yeast maceration juice [Lundsgaard, 1930, 2]. The action of this salt was also investigated by Yamazaki [1930], who pointed out that its inhibitory effect on the fermentation of hexosediphosphate by maceration juice was greater in the presence of arsenate, but came to the conclusion that the main effect was an inhibition of the esterifying power with phosphate. It seemed of interest to determine the effect of mono-iodoacetate on fermentation of hexosediphosphate by the hexosephosphatase referred to above, so that the process might be uncomplicated by the presence of zymase.

A hexosephosphatase was prepared by the autolysis of dried baker's yeast,

as previously described in detail [Macfarlane, 1930]. The preparation contained no co-enzyme and did not esterify or ferment sugar with added co-enzyme. A typical experiment was carried out as follows. Exp. 1. 1 cc. of a 20 % solution of the enzyme, 2 cc. 0·1 *M* potassium hexosediphosphate and 1 cc. boiled yeast extract were placed in each of four test-tubes, to which were added:

(*a*) 2 cc. H_2O;
(*b*) 0·6 cc. 1 % potassium mono-iodoacetate + 1·4 cc. H_2O;
(*c*) 0·3 cc. 0·1 *M* Na_2HAsO_4 + 1·7 cc. H_2O;
(*d*) 0·3 cc. 0·1 *M* Na_2HAsO_4 + 0·6 cc. potassium mono-iodoacetate + 1·1 cc. H_2O.

The tubes were placed in a thermostat at 30°, 1 cc. samples being withdrawn at intervals and the inorganic P estimated by the Briggs method in the trichloroacetic acid filtrates. The quantities of phosphate liberated in 30 minutes, as mg. P per cc., were in (*a*) 0·175; in (*b*) 0·160; in (*c*) 0·984; and in (*d*) 0·551. The effect is more strikingly shown by plotting the rates of liberation of inorganic P (corrected for the change occurring in the absence of co-enzyme) over short time intervals, as in Exp. 2 (Fig. 1). The curve (*b*) obtained

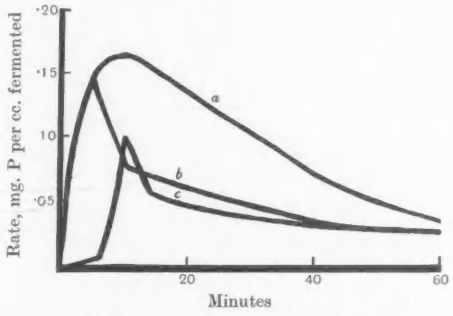

Fig. 1. Fermentation of hexosediphosphate.

Exp. 2. (*a*) 2 cc. 10 % hexosephosphatase + 5 cc. 0·1 *M* hexosephosphate + 2 cc. yeast extract + 6 cc. 0·1 *M* As_2O_5 + 2·4 H_2O.
(*b*) 2 cc. 10 % hexosephosphatase + 5 cc. 0·1 *M* hexosephosphate + 2 cc. yeast extract + 6 cc. 0·1 *M* As_2O_5 + 1·4 cc. 1 % mono-iodoacetate + 1 cc. H_2O.
(*c*) 2 cc. 10 % hexosephosphatase + 5 cc. 0·1 *M* hexosephosphate + 2 cc. yeast extract + 6 cc. 0·1 *M* As_2O_5 + 0·6 cc. 1 *M* NaF + 1·8 cc. H_2O.

with mono-iodoacetate is almost identical with (*a*), the control with arsenate, for the first 5 minutes and then rapidly falls to a low rate coinciding with that reached by the curve (*c*) representing a similar fermentation in presence of fluoride. The decrease in rate shown in curve (*a*) after 20 minutes is due to the decreasing concentration of hexosediphosphate, 50 % of which has been decomposed at this point.

The nature of the ester remaining in the present case has not been ascertained but in view of the similarities already noticed between fluoride and mono-iodoacetate it appears probable that the reason for the inhibition of

fermentation lies not so much in a direct effect on the enzyme as in the fact that it is now acting on a less congenial substrate.

The effect of potassium mono-iodoacetate on esterification and fermentation by yeast juice.

Unlike maceration juice, which was used by Lundsgaard, yeast juice contains a fair amount of glycogen and a distinction has therefore to be drawn between the esterification of added sugar and that of the glycogen already present in the medium.

Autofermentation of yeast juice. Flasks containing the desired mixtures were placed in a thermostat at 30°, the CO_2 evolved being measured. Samples for phosphate estimation were taken at zero time and subsequently at intervals. After fermentation had been allowed to proceed for an hour, arsenate was added to make a final concentration of 0·01 *M*. The course of the esterification and fermentation is illustrated in Table I and Fig. 2.

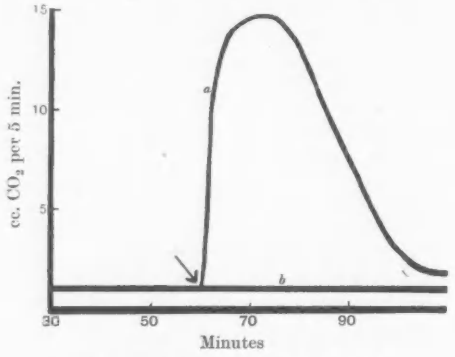

Fig. 2. Autofermentation of yeast juice.
(*a*) Control. (*b*) +mono-iodoacetate. The arrow marks addition of 3 cc. 0·1 *M* arsenate.

Table I.

(*a*) 20 cc. yeast juice + 3 cc. 0·6 *M* K_2HPO_4 + 3 cc. H_2O.
(*b*) 20 cc. yeast juice + 3 cc. 0·6 *M* K_2HPO_4 + 3 cc. 2 % mono-iodoacetate.

	(*a*)		(*b*)	
	mg. P esterified	CO_2 as mg. P ($CO_2 \equiv P$)	mg. P esterified	CO_2 as mg. P ($CO_2 \equiv P$)
1st hr.	+41·5	21·4	+33·6	20·7
2nd hr. (with arsenate)	−89·6	91·5	+12·5	10·6

The autofermentation of the juice is considerable, but is slower than the fermentation of sugar and is not appreciably diminished by mono-iodoacetate. A slow esterification of phosphate takes place, which is more than equivalent to the CO_2 produced; in presence of 0·1 *M* iodoacetate 80 % of this esterification still occurs. On adding arsenate the rate of CO_2 evolution in the normal juice

increases considerably for a time and then returns nearly to its original level the excess CO_2 being almost exactly equivalent to the amount of esterified P which disappears during this period. This confirms Meyerhof's [1927] statement that the accelerated rate in presence of arsenate in glycogen fermentation is due entirely to the increased rate of fermentation of accumulated hexosediphosphate and not to an acceleration of the diastatic activity of the juice.

In the presence of mono-iodoacetate no acceleration takes place with arsenate, although the concentration of esterified P is of the same order as that in the control juice at this point; the esterification of phosphate however continues, 12·5 mg. being esterified in the next hour. The iodoacetate has therefore inhibited the acceleration by arsenate without greatly affecting the esterifying power with regard to glycogen.

Fermentation of sugar by yeast juice. On adding phosphate and mono-iodoacetate simultaneously to yeast juice fermenting sugar, an immediate esterification of phosphate occurs, accompanied by an equivalent evolution of CO_2, 90 % esterification taking place as compared with the control juice. The basal rate of fermentation, however, quickly falls and is not accelerated by the addition of arsenate, while on subsequently adding phosphate no rapid esterification and fermentation take place. A slow esterification of phosphate continues still, corresponding approximately to the rate of glycogen esterification. The esterification of sugar by yeast juice is therefore inhibited by a concentration of mono-iodoacetate which only slightly affects that of glycogen.

The course of a typical experiment is shown in Table II.

Table II.

(*a*) 20 cc. yeast juice + 3 cc. 0·6 *M* K_2HPO_4 + 2 g. fructose + 3 cc. H_2O.
(*b*) 20 cc. yeast juice + 3 cc. 0·6 *M* K_2HPO_4 + 2 g. fructose + 3 cc. 2 % mono-iodoacetate.

	(*a*)		(*b*)	
	mg. P esterified	CO_2 as mg. P ($CO_2 \equiv P$)	mg. P esterified	CO_2 as mg. P ($CO_2 \equiv P$)
30 min.	+47·4	45·0	+43·3	20·7
1 hr. 10 min. (with arsenate and phosphate)	+ 3·0	555·0	+15·2	32·0

The salt acts in a manner similar to fluoride, though its effect is not so immediately apparent. The direct fermentation of hexosediphosphate either by yeast juice or by the hexosephosphatase preparation and co-enzyme is also inhibited as shown by the failure to respond to the presence of arsenate by an increased rate.

The results quoted above afford additional confirmation of the theory of Meyerhof [1927] that a direct fermentation of hexosediphosphate takes place in fermenting yeast preparations and in muscle extracts, in addition to the co-enzyme-free hydrolysis described by Harden and Young [1910]. The possibility is not excluded that a labile sugar may be formed by hydrolysis of

hexosediphosphate in presence of co-enzyme, capable of fermentation without previous re-esterification, rather than a disruption of the molecule with the simultaneous production of phosphate and some intermediate compound, *e.g.* methylglyoxal; moreover, although in the experiments here recorded with the hexosephosphatase preparation the accelerated decomposition of the ester in presence of arsenate was always accompanied by evolution of CO_2, it is possible that this may not hold for other yeast preparations.

II. The action of arsenate on the induction period of zymin.

It has been shown [Harden, 1925; Harden and Macfarlane, 1928] that mixtures of zymin and sugar solution normally show an induction period varying in duration with the volume of solution added before the onset of rapid fermentation occurs, and that arsenate increases the length of this period during which no evolution of CO_2 takes place. It was noticed that the inorganic phosphate present in trichloroacetic acid filtrates from the mixtures increases during the induction period. If this phosphate arises from a fermentation of hexosediphosphate, the rate of liberation should increase on addition of arsenate and simultaneously, since no CO_2 is evolved, an accumulation of the hexose residue, either unchanged or in the form of an intermediate product, should occur. If, on the other hand, the phosphate is not increased by the addition of arsenate, it must be derived either from a hydrolysis of hexosediphosphate or from some other change in the phosphorus compounds present.

Duplicate series of flasks were prepared containing 2 g. of zymin and known volumes of sugar solution or water; to one arsenate was added in the desired concentration (0·02–0·002 *M*), the corresponding flask of equal volume acting as the control. In some cases hexosediphosphate was added to shorten

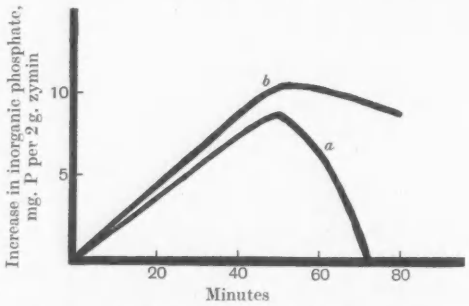

Fig. 3. Induction period of zymin fermentation.
(*a*) 2 g. zymin + 40 cc. H_2O. Induction time, 80 min.
(*b*) 2 g. zymin + 8 cc. 0·1 *M* Na_2HAsO_4 + 32 H_2O. Induction time, ∞.

the induction period. The flasks were placed in a thermostat at 30° and the inorganic phosphate was estimated by the Briggs method in the trichloroacetic acid filtrates of samples taken at frequent intervals. Figs. 3 and 4 illustrate typical experiments.

It was found that the maximum liberation of inorganic phosphate took place in the larger volumes employed, that is where the liberation of the available phosphate was completed before any esterification of phosphate prior to fermentation developed, the rate of liberation being linear and unaltered by the addition of arsenate. As the volume decreased, the initial rate of increase was still linear but first gradually and then abruptly decreased as esterification was established, the middle portion of the curve (Fig. 3 *a*) representing the balance between a constant liberation and an increasing esterification. The addition of arsenate did not increase the rate of liberation

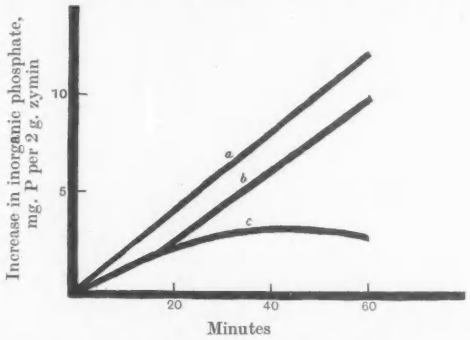

Fig. 4. Induction period of zymin fermentation.

(*a*) 2 g. zymin + 1 g. fructose + 60 cc. H_2O. Induction time, > 80 min.
(*b*) 2 g. zymin + 1 g. fructose + 2·4 cc. 0·5 *M* Na_2HAsO_4 + 2·5 cc. 0·1 *M* K hexosediphosphate + 55·1 H_2O. Induction time, > 3½ hr.
(*c*) 2 g. zymin + 1 g. fructose + 2·5 cc. hexosediphosphate + 57·5 H_2O. Induction time, 0.

(Fig. 4, *a* and *b*), though by its effect in delaying esterification and thus maintaining the original linear rate, the total amount of the increase in inorganic phosphate was greater than in the control in those cases where the normal induction time was shorter than the time of complete hydrolysis. Estimation of methylglyoxal by distillation and precipitation as the *p*-nitrophenylosazone showed that no accumulation of triose had taken place during the induction period.

It was also found that the total amount of acid-soluble phosphorus present in the trichloroacetic acid filtrate rose during the induction period by an amount approximately equal to the increase in inorganic phosphate. Thus in one case the inorganic phosphate increased from 9·13 to 19·4 mg. during an induction period of 60 minutes, while the total acid-soluble P increased from 23·2 to 32·0 mg. It seems probable that the increase in inorganic phosphate must be due to changes in some other phosphorus-containing substance in zymin during the induction period rather than to the decomposition of hexosephosphoric esters. It must at least be concluded that the action of arsenate in prolonging the induction is not directly due to its specific action in accelerating the rate of fermentation of hexosediphosphate.

It is interesting to note that the same treatment which decreases the rate of fermentation of yeast and enables it to acquire the power of responding to phosphate, such as drying or treating with acetone, also injures the indophenol oxidase-cytochrome system [Keilin, 1929], one of the instances of injury being that cytochrome in dried yeast and zymin is reduced in amount and undergoes oxidation and reduction more slowly than in untreated yeast. In yeast suspensions warmed to 52° the reduction of cytochrome is markedly accelerated by lactate and also by succinate [Keilin, 1929], while arsenate increases the reduction time in suspensions of fresh yeast [Bierich and Rosenbohn, 1929]. The antagonistic action of these two salts in this case is parallel with their effect on the induction period of zymin fermentation, which is decreased and prolonged by lactate and arsenate respectively [Harden and Macfarlane, 1928]. This indicates that the phenomenon may be associated with the cytochrome content of the yeast preparation, and attempts are being made to obtain experimental evidence on this question.

Summary.

1. The fermentation of hexosediphosphate by the hexosephosphatase preparation previously described in presence of co-enzyme and arsenate is inhibited by mono-iodoacetate.

2. The esterification of sugar by yeast juice is inhibited by mono-iodoacetate in concentrations which only slightly inhibit that of glycogen.

3. Arsenate does not accelerate the rate of fermentation by yeast juice in presence of mono-iodoacetate.

4. The rate of liberation of inorganic phosphate during the induction period of zymin fermentation is not accelerated by arsenate, showing that this phosphate does not arise by fermentation of hexosediphosphate.

I wish to record here my gratitude to Prof. A. Harden for his constant help and advice throughout this work.

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XCIV. THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE PRECIPITATING AND PROTECTIVE ACTIONS OF PROTEINS ON COLLOIDAL GOLD AND GUM BENZOIN.

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In a previous communication [Wright and Kermack, 1923, 1], evidence has been adduced that the power of gelatin to protect colloidal gum benzoin against precipitation by sodium chloride depends on the p_H and is minimal at the isoelectric point. The variation of protection with p_H was small and so it appeared desirable to confirm the results and to extend the observations to other proteins and to another lyophobic colloid. The present communication deals primarily with the protective action of gelatin, ovalbumin, serum-albumin, the serum-globulins, oxyhaemoglobin and edestin on colloidal gum benzoin and colloidal gold at various hydrogen ion concentrations. The effect of variation of p_H on the protective power of colloids is of interest not only from the theoretical point of view, but also because such variations may be of importance in biological systems and in technical processes.

The opportunity was taken to make further observations on the action of the proteins on the lyophobic colloids in the absence of salt. It was shown by Wright and Kermack [1923, 1, 2] that colloidal gum benzoin was precipitated by very small concentrations of gelatin, serum-globulin and oxyhaemoglobin at reactions on the acid side of the isoelectric point, and by somewhat higher concentrations at reactions approaching or corresponding with the isoelectric point [see also Kermack and McCallum, 1924]. Michaelis and Nakashima [1923] almost simultaneously, working with mastic sol, developed a method for the determination of the isoelectric point of proteins in which use was made of the same principle, and in this way obtained values for the isoelectric points of the serum-albumins of various animals. Reinders and Bendien [1925, 1928] have also investigated the reaction between lyophilic and lyophobic colloids at various reactions, and these authors observed that colloidal gold was coagulated by small concentrations of gelatin on the acid side of the isoelectric point. Similar observations were made with other proteins, caseinogen and sodium lysalbuminate. They observed that at p_H 6

or higher all the proteins used exerted no precipitating but only protective action, but the precise effect of variation of p_H on the latter does not appear to have been closely investigated. Especially in view of the proposal of Michaelis and Nakashima to use the phenomenon as a method for the determination of isoelectric points, it seemed desirable to enquire more closely into its general application to various proteins and lyophobic colloids.

Experimental.

The colloidal gum benzoin was prepared as described by Wright and Kermack [1923, 1, p. 637], and the colloidal gold by adding to 100 cc. of distilled water 1 cc. of 1 % solution of photographic gold chloride, 1 cc. of a 1·4 % solution of potassium carbonate (previously dried and ignited), heating to boiling point, and then adding with vigorous shaking, after removal from the flame, 1 cc. of a solution of neutralised 0·40 % formaldehyde.

To each of a series of tubes were added 0·25 cc. of protein of appropriate concentration, 0·5 cc. of acid or alkali or buffer mixture as the case may be, and 1·0 cc. of gold or benzoin sol. After mixing, the tubes were allowed to stand overnight, when, if desired, readings were made in order to record the interaction between the protein and sol in the absence of salt. 0·25 cc. of sodium chloride (10 %) was now added and the tubes shaken and read after a further 24 hours' standing. The readings were recorded in tables similar to those given in previous papers [Wright and Kermack, 1923, 1, 2], and from these readings figures were prepared showing the regions in which complete or almost complete precipitation takes place. To save space, only the figures are given. In the case of the gold, in certain tubes the precipitate was red or purple, and not blue. The regions in which red precipitates were obtained are shown by shading. All the protein concentrations given refer to the final concentrations. As the readings in the absence of salt were made when the volume was 7/8 of the volume after the addition of the salt, the concentrations of protein should really be 8/7 of those given, but as the protein concentrations were doubled in consecutive tubes, the correction required is insignificant.

In the case of colloidal gum benzoin, which itself has a p_H of about 3·8, the various organic acids present in low concentration have a sufficient buffering effect to stabilise the p_H over the range employed in these experiments. In the case of colloidal gold, however, it was usually necessary to employ small concentrations of buffer mixtures in order to obtain satisfactory results. The concentration of buffers was small ($N/200$) in comparison with that of the sodium chloride employed to effect the precipitation, so that any effect of the ions of the buffer in sensitising or protecting the gold would be small in comparison with the effect of the sodium and chlorine ions. The exact buffers used are stated in the figures. The p_H measurements were usually made colorimetrically by means of the B.D.H. capillator, and in some cases confirmed by means of the quinhydrone electrode.

It is to be emphasised that the transition from complete protection to complete precipitation is usually a gradual one, and that, further, in this transition region the exact amount of precipitation depends on the precise conditions of the experiment. The aim of these experiments was, therefore, to determine broadly the approximate conditions under which precipitation and protection take place, and the figures should be interpreted accordingly.

Gelatin. Isoelectric gelatin was prepared according to the method of Loeb [1922, p. 35], and the protein content determined by drying to constant weight at 100°. Dilutions in distilled water were prepared and the experiment carried out as described above. The results are shown in Figs. 1 A and 1 B.

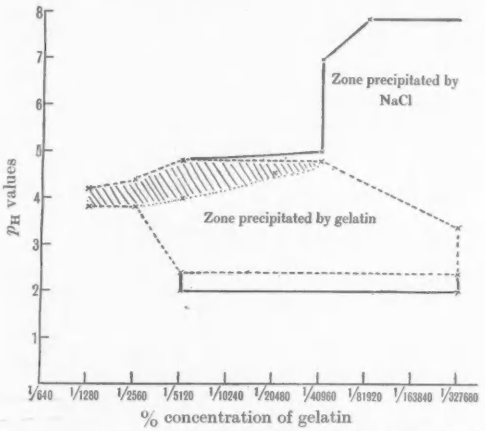

Fig. 1 A. Action of gelatin on gold sol. Final concentration of buffer used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of gelatin p_H 4·7.

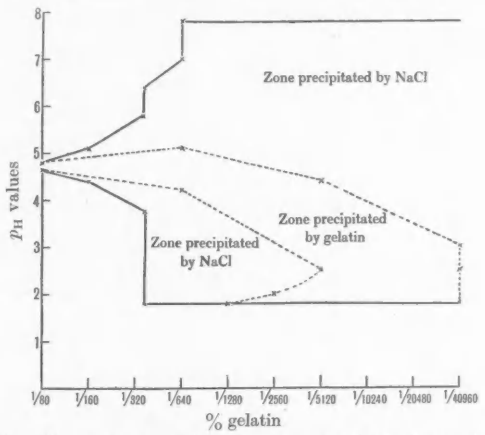

Fig. 1 B. Action of gelatin on gum benzoin. No buffers used.

Ovalbumin. Crystalline ovalbumin (Merck), 1 g. in 100 cc. of distilled water, was filtered to remove a small amount of flocculent insoluble material, and the requisite dilutions in distilled water prepared. The results are shown in Figs. 2 A and 2 B.

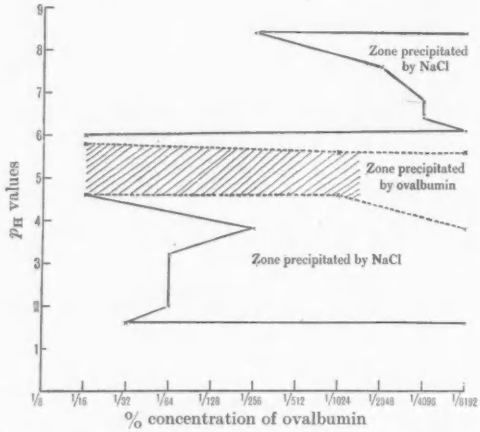

Fig. 2 A. Action of ovalbumin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of ovalbumin p_H 4·6–4·8.

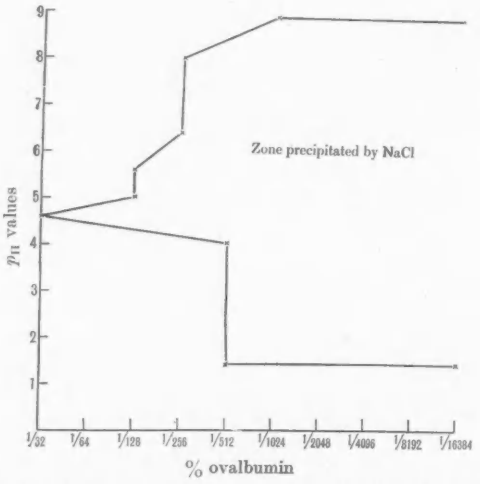

Fig. 2 B. Action of ovalbumin on gum benzoin. No buffers used.

Serum-albumin, pseudoglobulin and euglobulin. A quantity of human plasma, separated from oxalated blood, was fractionated by ammonium sulphate precipitations. The fibrinogen was removed by quarter saturation and

the serum-globulins in the filtrate precipitated by half saturation, filtered and washed with half-saturated ammonium sulphate. The albumin was precipitated by full saturation. Each fraction was dissolved in distilled water, reprecipitated, washed, redissolved in water and dialysed through a collodion membrane until practically free from sulphate. The euglobulin, which had precipitated during dialysis, was separated from the pseudoglobulin and was dissolved in 0·75 % sodium chloride solution. The protein present in each solution was determined by means of the Folin-Wu method as in the determination of serum-proteins.

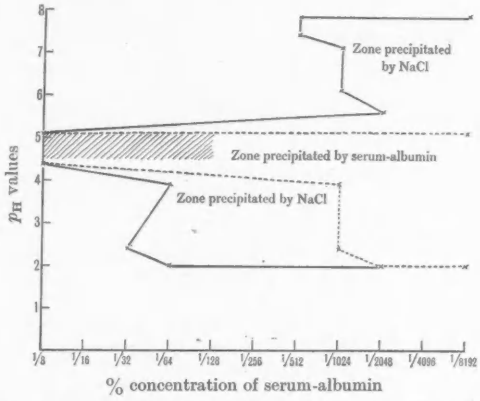

Fig. 3 A. Action of serum-albumin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of serum-albumin p_H 4·7–4·9.

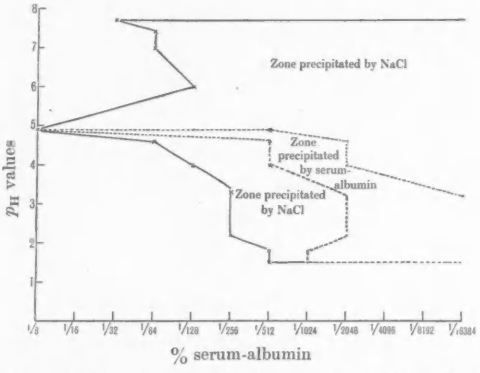

Fig. 3 B. Action of serum-albumin on gum benzoin. No buffers used.

The results appear in Figs. 3 A and 3 B (serum-albumin), 4 (serum-pseudoglobulin) and 5 (serum-euglobulin). In the case of euglobulin the original solution necessarily contained sodium chloride required to effect solution,

but the dilutions were made with distilled water so that in the lower concentrations of protein the concentrations of salt were correspondingly reduced.

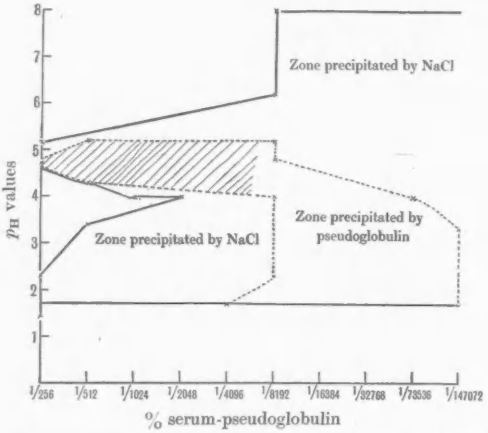
Zone precipitated by NaCl
Zone precipitated by NaCl
Zone precipitated by pseudoglobulin
p_H values
1/256 1/512 1/1024 1/2048 1/4096 1/8192 1/16384 1/32768 1/73536 1/147072
% serum-pseudoglobulin

Fig. 4. Action of serum-pseudoglobulin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of serum-pseudoglobulin p_H 4·7–4·8.

In any case the final concentration of salt added was considerably greater than that present in the most concentrated protein solution used, so that,

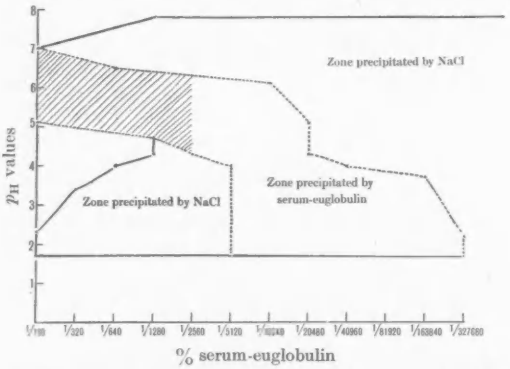
Zone precipitated by NaCl
Zone precipitated by NaCl
Zone precipitated by serum-euglobulin
p_H values
1/160 1/320 1/640 1/1280 1/2560 1/5120 1/10240 1/20480 1/40960 1/81920 1/163840 1/327680
% serum-euglobulin

Fig. 5. Action of serum-euglobolin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of serum-euglobulin p_H 5·5.

although the concentration of salt in the final mixture was not constant, the variations were relatively small.

Oxyhaemoglobin. Human red blood corpuscles, centrifuged from oxalated plasma were washed several times with 0·9 % salt solution, mixed with distilled water to cause haemolysis and repeatedly centrifuged to remove stromata. The concentration of oxyhaemoglobin in the dialysed solution was

determined by using the Zeiss stuphophotometer to ascertain the extinction coefficient, the filter S 51 ($\mu\mu$ 490–530) being employed. The concentration of haemoglobin in a particular solution was found by the Kjeldahl method (total nitrogen), and from the extinction coefficient of this solution it was calculated that a 3 cm. layer of a 0·01 % solution had an absorption coefficient of 0·1358. The percentage concentration of any solution is therefore given by the formula $x = \frac{0{\cdot}01\sigma}{0{\cdot}1358}$, where σ is the observed extinction coefficient for a 3 cm. layer.

Dilutions in distilled water were prepared and the experiments carried out in the usual manner. Owing to the colour of oxyhaemoglobin it was difficult or impossible to make readings of the precipitation of gold in the ordinary way. It was found, however, that by observing the tubes in a beam from an arc lamp, the presence of particles of colloidal gold throughout the solution could readily be detected by the Tyndall effect. Tubes in which sedimentation of gold had not occurred gave a marked scattering of incident light. In the experiments with gum benzoin this difficulty did not arise, and readings could be easily made in the ordinary way. The results are given in Figs. 6 A and 6 B. It was found that in the tubes in which the p_H was 5 or

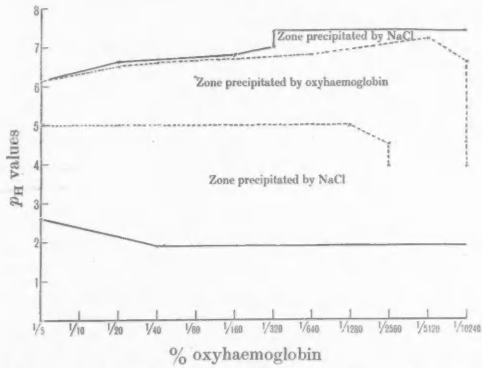

Fig. 6 A. Action of oxyhaemoglobin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of oxyhaemoglobin p_H 6·7–6·8.

less, a brown colour developed, clearly due to the splitting of the oxyhaemoglobin into haematin and globin. The results in this region should therefore be interpreted with caution.

Edestin. Several attempts were made to carry out experiments with edestin from hemp seed (Merck), but no satisfactory results could be obtained, owing to the very small solubility of this protein in distilled water in the neighbourhood of the isoelectric point. In the most concentrated solutions prepared no protection of gold or benzoin was observed between p_H 3·5 and 7·5. Above and below this range protection was observed with 0·05 % edestin. In another experiment in which, in order to obtain higher concentrations of edestin, the

protein was dissolved in 5 % sodium chloride and the solution adjusted to various reactions from p_H 3·0 to 8·5 by the addition of a suitable acetate-phosphate buffer mixture ($M/50$), no protection was observed when an equal volume of gold or benzoin sol was added to these mixtures. The final concentration of edestin in these experiments ranged from 0·1 to 0·0002 %, that of

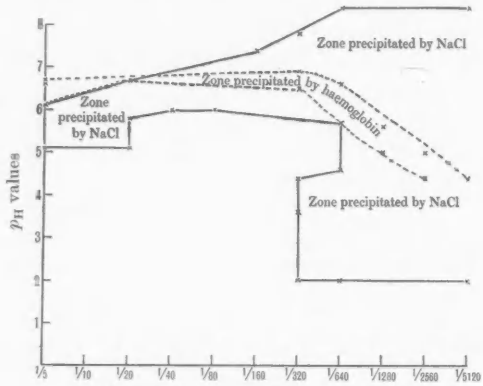

Fig. 6 B. Action of oxyhaemoglobin on gum benzoin. No buffers used.

salt being constant throughout. The gold in all the tubes was completely precipitated, but, in the more concentrated solutions and the more alkaline of these, the precipitates were distinctly red and graded steadily to blue, being quite blue in the dilute solutions (all reactions) and in acid solutions (all concentrations).

Discussion.

As the results obtained with oxyhaemoglobin are in some respects anomalous, these will be discussed separately after dealing with the other proteins.

Precipitation in the absence of added electrolytes (excluding the experiments with oxyhaemoglobin). The observations are in agreement with those previously made by Wright and Kermack [1923, 1] and Michaelis and Nakashima [1923]. In the presence of small quantities of proteins on the acid side of the isoelectric point, and therefore positively charged, the discharge of the negative colloid takes place and precipitation occurs. When the concentration of protein is very low, precipitation is observed only at reactions well on the acid side of the isoelectric point, as otherwise the protein adsorbed on the negative colloid bears an insufficient number of positive charges to discharge the colloid. As the concentration of protein is increased the p_H at which precipitation occurs approaches more nearly to the isoelectric point. It is not to be expected that the limit p_H at which precipitation occurs will always coincide exactly with the isoelectric point, as it depends for example on such

factors as the degree of adsorption of the protein on the particles, the rate at which ionisation increases as the isoelectric point is left, and the protective power of the protein. If this latter were sufficiently great, it might prevent precipitation at a concentration of protein insufficient to bring the zone of precipitation up to the isoelectric point. There is also the possibility that the isoelectric point of the protein might be altered by adsorption, for example as the result of denaturation. Further, if we are to compare the isoelectric point of the protein with the limiting p_H at which precipitation occurs when the concentration of protein is increased, it is necessary to define the latter unambiguously. In the case of gelatin and mastic, or gelatin and gum benzoin, there is no ambiguity, as when the concentration of protein is increased the zone of precipitation narrows down and converges on a particular point which is not, however, necessarily the maximum value of p_H at which precipitation occurs. With lower concentrations of protein precipitation may occur at a higher p_H, although the mean p_H of the zone obtained at any particular protein concentration does not exceed, but tends to approach, the limiting p_H (see Fig. 1 B). In the case of a protein which, in the neighbourhood of the isoelectric point, brings about precipitation, even at high concentrations, it is clear that the mean p_H of the zone must be taken. The actual width of the zone itself at a given protein concentration depends on various factors, *e.g.* the concentration of buffer used, or the stability of the lyophobic colloid, but the mean p_H is relatively constant. We shall therefore consider the "limit mean" p_H of the zone in relation to the isoelectric point. It appears from the diagrams that when colloidal gum benzoin is used, this "limit mean" p_H does in fact approximately agree with the isoelectric point, but when gold is employed the agreement is not so good, and in the case of oxyhaemoglobin there is a discrepancy of more than one unit of p_H.

Another point which should be noted in connection with the precipitation of gold is that the precipitates were not infrequently red or purple in colour instead of blue. As the blue colour of gold precipitated by inorganic salts may be taken as an indication of the adhesion of the gold particles together, it would seem that the purple or red colour of these precipitates indicates that the gold particles were still virtually separate. The red precipitate showed itself chiefly in the immediate neighbourhood of the isoelectric point and in the presence of moderate concentrations of protein. It would appear that the protein micelles become aggregated round the gold particles and, as a result of this, they sediment relatively more easily. When the isoelectric point is reached and the charge on the protein virtually disappears, these aggregates, each containing a gold particle, may sediment, even although the pure protein is quite stable under these conditions. The gold particles will, however, be carried down in a dispersed condition, and a red precipitate will be formed. The zone of red precipitation was especially wide in the case of serum-euglobulin, and this is in agreement with the fact that this protein is relatively easily precipitated from solution.

Protection in the presence of electrolytes (excluding the experiments with oxyhaemoglobin). On the addition of salt (sodium chloride) any particles of the lyophobic colloid which have adsorbed no protein or only very small amounts will tend to lose their charges and will then be free to aggregate, and in the case of gold the colour will change to blue. Salt, in the concentration employed in these experiments, will have comparatively little direct effect on the proteins. It is not to be expected that the red region of precipitation will be markedly extended, and, further, the entanglement of the particles in the precipitated protein where this has occurred will prevent the change of colour to blue. The charge of the particles which are surrounded with protein will depend primarily on the relation of the p_H of the medium to the isoelectric point of the protein. Disregarding for the moment the tubes in which red precipitation occurred before the addition of salt, it appears in general that the region of blue precipitation occurs in more and more concentrated protein as the isoelectric point is approached. To this extent there is evidence that the protective action of the protein decreases in the neighbourhood of the isoelectric point. We may as a working hypothesis assume that the amount of adsorption of the protein is, at a first approximation, independent of the hydrogen ion concentration in the neighbourhood of the isoelectric point, and that an approximately similar film is formed whatever the reaction. At some distance from the isoelectric point, the protective effect of this film is reinforced by the charge which it carries. As the isoelectric point is approached this charge decreases, and so the apparent protective power is less. At the isoelectric point itself the charge is zero just as it was before the addition of salt, so that no change in the stability is effected by the addition of salt at this point. This accords with experiment except in the case of haemoglobin.

In the neighbourhood of the isoelectric point, approximately the same results are obtained with benzoin as with gold, except that in this case the precipitates are white in every instance, and there is no apparent difference corresponding to that of red and blue precipitates with gold. Protection is minimal at the isoelectric point and increases on each side. At some distance on each side gum benzoin differs rather remarkably from gold in one respect. With gold the maximum amount of protection on the alkaline side is much greater than on the acid side; with gum benzoin, on the other hand, approximate equality on each side was observed. Thus, for example, with serum-albumin at p_H 6·0 to 6·1 protection was observed at dilutions of 1/512 % and 1/64 % with gold and gum benzoin respectively, whereas at p_H 2·4 the corresponding dilutions were 1/16 % and 1/128 % respectively. It appears from these results that at p_H 2·4 eight times as much serum-albumin is required to protect gold as is required to protect gum benzoin, whereas at p_H 6·1 only one-eighth as much is required. It is rather difficult for any satisfactory explanation to be advanced, but it would appear that in acid solution there is relatively little adsorption of the positively charged protein ions by the gold.

Precipitation and protection by oxyhaemoglobin. The reaction between oxyhaemoglobin and gum benzoin in the absence of salt has already been described by Wright and Kermack [1923, 1], who used sheep's oxyhaemoglobin, and these results have been confirmed using human oxyhaemoglobin, the "limit mean" p_H found in this case being about 6·6. After the addition of sodium chloride the absence of protection was observed between p_H 5·4 and 6·0. The zone of minimum protection is thus distinctly to the acid side of the isoelectric point, which is about 6·8 [Michaelis and Airila, 1921; Geiger, 1931]. A cataphoretic experiment in a U-tube with the oxyhaemoglobin employed in the precipitation experiments showed that it was in fact positively charged at p_H 6·3. It was very curious to find that, on the addition of salt, partial or complete re-dispersion occurred in certain tubes at p_H 6·0 to 6·6. The results in the case of gold were even more unexpected. In the absence of salt the "limit mean" p_H at which precipitation occurs is about p_H 5·8 or even lower. When very little buffer was present, precipitation extended from p_H 5·0 to 5·5 in the higher concentrations of oxyhaemoglobin, and from p_H 5·0 to 6·0 in the lower concentrations. In an experiment carried out with phosphate buffers added to control p_H a widening of this zone occurred, but in the higher concentrations of protein, no precipitation took place above p_H 6·2, although at lower concentrations precipitation was observed from p_H 4·6 to 7·2. It is clear that, in the case of gold, the centre of the zone of precipitation is well below the isoelectric point, and without added buffers the whole zone may be well below it. When salt is added, very little change occurs on the alkaline side of the zone, except a widening in very low concentrations, but on the acid side extensive precipitation occurs and there is practically no protection. It has, however, to be remembered that the zone in the absence of salt extends down to p_H 5·0, and that below this p_H dissociation of oxyhaemoglobin into its components takes place, so that too much significance should not be attached to these observations below p_H 5·0.

It is clear that with this protein distinct abnormalities occur; in particular, it sometimes behaves as though its isoelectric point were well below the generally accepted value. There is no doubt that the gold particles in presence of haemoglobin or other proteins are positively charged in moderately acid and negatively in moderately alkaline solution. In the case of proteins other than oxyhaemoglobin, the zone of precipitation coincides with a zero charge on the protein, so that, on the acid side of this zone the particles are positively charged and on the alkaline side negatively charged. When the zone of precipitation coincides with the isoelectric point, this is entirely reasonable. But when the zone of precipitation is on the acid side, it follows that between this zone and the isoelectric point the particles must be negatively charged whilst the protein itself is positively charged. An experiment in which an attempt was made, by means of the ultramicroscope, to observe the charge on the gold particles in the presence of oxyhaemoglobin at p_H 6·3, indicated that it was in fact negative, but as it was relatively small, it was

difficult to ascertain its sign with absolute certainty. As mentioned above, a cataphoretic experiment by the U-tube method on the oxyhaemoglobin alone, showed that this protein had a positive charge at this p_H. Reference to previous work [Wright and Kermack, 1923, 1, 2; Kermack and McCallum, 1924; Michaelis and Nakashima, 1923] makes it clear that a region in which the particles are negatively charged while the protein is positively charged normally exists when the concentration of protein is sufficiently low, for in this case the zone of precipitation lies well below the isoelectric point. The explanation in this instance is clearly that the protein, being present in low concentration, must be at a p_H well below the isoelectric point in order that it should be sufficiently positively charged to discharge the negatively charged lyophobic particles completely. It is possible, then, that the abnormalities observed with oxyhaemoglobin may be due to the small adsorption of this protein on the colloidal particles in the neighbourhood of the isoelectric point. If only small adsorption takes place, it is possible for the positively charged protein to exist in stable equilibrium with negatively charged particles.

An alternative explanation might be based on the observation that in certain cases proteins are denatured when adsorbed on a surface [Loeb, 1923] and that the isoelectric point of the denatured protein might differ from that of the native protein. It might be that the adsorbed oxyhaemoglobin is denatured and now possesses an isoelectric point about one unit of p_H below that of the native protein. This hypothesis, however, appears to be untenable, in the first place, because it does not explain the difference in the "limit mean" p_H values in the cases of gold and of benzoin, and in the second place, as the result of experiments made to determine the isoelectric point of oxyhaemoglobin denatured by heating to 90° for 5 minutes. By means of ultramicroscopic observations it was found that the particles had a positive charge at p_H 6·0, and a barely detectable positive charge at p_H 6·4. At p_H 6·5 and at p_H 7·0 no charge was discernible, whilst at p_H 7·5 the particles bore a small but negative charge. Heat-denaturation is of course not necessarily the same as surface denaturation, but the above two considerations appear to render the explanation unlikely.

It may be noted that, when a low concentration of calcium chloride (0·05N) was used in place of the usual concentration of sodium chloride employed in the experiments with colloidal gold and oxyhaemoglobin, a somewhat different result was obtained. The effect of the addition of calcium chloride was to extend the zone of precipitation on the alkaline as well as on the acid side. This fits in with the view that the particles of gold covered with oxyhaemoglobin are negatively charged at p_H 6·0 or more, as in that case the bivalent calcium ions would in this region effect precipitation more readily than the univalent sodium ions.

General. These results confirm the previous findings reported by Wright and Kermack [1923, 1] and almost simultaneously by Michaelis and Nakashima [1923] to the effect that, when low concentrations of protein are employed,

a zone of precipitation occurs on the acid side of the isoelectric point, and that, as the concentration of protein is increased, this zone approaches the isoelectric point, but does not pass it. As mentioned in the introduction, Michaelis and Nakashima have gone so far as to suggest that these observations form the basis of a method for the determination of the isoelectric point of soluble proteins, and have applied it in the case of a number of serum-albumins. It is to be observed, however, that they had apparently tested out the principle only over a limited range, namely with gum mastic and gelatin, and it is doubtful, in view of the variability of the isoelectric point of gelatin, how far their result, namely p_H 4·65, can be considered as a decisive proof of the validity of the method. The results now obtained with oxyhaemoglobin and gold show that, at least when certain lyophobic colloids are used, quite erroneous results may be obtained. Thus, although there is little doubt as to the general nature of the phenomena observed, there does not seem to be sufficient basis for applying the principle without qualification, as suggested by Michaelis and Nakashima, to the quantitative determination of the isoelectric points of proteins.

Summary.

1. Observations have been made on the precipitation of colloidal gold and gum benzoin by gelatin, ovalbumin, serum-albumin, pseudoglobulin, euglobulin and oxyhaemoglobin at different concentrations of protein and of hydrogen ions. The observations confirm and extend the results previously obtained. In very low concentrations of protein, precipitation occurs on the acid side of the isoelectric point, but as the concentration of protein increases the zone of precipitation approaches the isoelectric point, and when sufficient protein is present, the centre of the zone, except in the case of oxyhaemoglobin, is at a point not significantly different from the isoelectric point.

2. In the presence of 1·25 % sodium chloride, minimum protection by these proteins, except oxyhaemoglobin, is observed at a reaction not significantly different from the isoelectric point. In the case of gold the precipitates observed in the neighbourhood of the isoelectric point are usually red in colour.

3. With oxyhaemoglobin abnormal results were obtained in respect both of precipitation and protection. This protein behaved as if its isoelectric point lay below its real value of about p_H 6·8. The anomaly is more pronounced in the case of gold than of gum benzoin. The explanation does not appear to depend on a change in the isoelectric point of the protein following on denaturation as a result of adsorption on the lyophobic particles, but may be due to small adsorption of the protein in the neighbourhood of the isoelectric point.

4. The results with oxyhaemoglobin and gold suggest that the method proposed by Michaelis and Nakashima for the determination of the isoelectric point of proteins is not of general application, and, in particular, may give erroneous results when gold is employed.

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XCV. STUDIES IN THE METABOLISM OF TISSUES GROWING *IN VITRO*.

IV. EFFECT OF FRUCTOSE, GALACTOSE AND XYLOSE UPON THE AMMONIA AND UREA PRODUCTION OF EMBRYO KIDNEY TISSUE.

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Previous work has shown that by using the tissue culture technique the production of ammonia and urea by growing and non-growing cultures can be estimated. By comparison with controls it has been found that the growing cultures produce considerable amounts of these substances whereas the non-growing ones do not [Holmes and Watchorn, 1927], and that the presence of glucose, while favouring the growth of the tissue, inhibits the production of ammonia and urea during this growth [Watchorn and Holmes, 1927]. It seemed that it might be of interest to compare the effect of other sugars with that of glucose, in order to determine whether the utilisation of a carbohydrate necessarily has this "protein sparing" effect, or whether it depends upon the type of carbohydrate utilised and thus upon the method of breakdown.

The use of the tissue culture method has much to recommend it for work of this type. In the first place, it is valuable to know that one is dealing with the metabolism of growth and activity, and not with autolytic processes, or with the metabolism of cells surviving under conditions which are likely to upset its normal balance. Even in tissue culture the conditions are unnatural, but they are not sufficiently so to prevent the continuation of growth in the embryonic tissue taken. By noting the effect upon this growth of various substances added to the medium and utilised by the cells, it is possible to judge whether the breakdown of these substances is favourable or unfavourable to the activity of the tissue. Thus, in the earlier work with glucose, we found that the presence of the sugar prevented the ammonia and urea formation which is otherwise characteristic of growth, and also that this did not inhibit the growth, but encouraged it.

Experimental.

As in previous work embryonic rat kidney tissue has been used, and the details of technique have been the same as those previously described [Holmes

and Watchorn, 1927, 1929; Watchorn and Holmes, 1927]. The cultures were made in pyrex flasks, and the conditions of culture of the growing and non-growing tissues were identical, except that the growing fragments were allowed to rest upon very fine wisps of cotton-wool, while in the non-growing preparations the fragments were floated in a shallow layer of the medium. It is known that fragments floating freely in a medium do not grow to any appreciable extent. When necessary the system of controls described in our first paper was used, but in the experiments dealing with ammonia and urea production it was usually sufficient to compare the growing tissues with the non-growing ones, as these latter never formed any ammonia or urea and therefore gave exactly the same result as the controls.

At least two kidneys were used in each flask, and in order to obtain large enough differences, it was necessary to use four when the utilisation of carbohydrate was estimated. As it was obviously important to use only kidneys taken from the same litter throughout each experiment, the number of preparations that could be set up each time was limited by the size of the litter. When the nitrogen metabolism of cultures with and without sugar was being compared, the media were both made from the same embryo extract and were equally diluted, in the one case with Ringer's solution containing the carbohydrate and in the other with plain Ringer's solution. 2 cc. of the medium were used in each flask, and the cultures were incubated for 2 days.

The solutions of carbohydrate were sterilised by passing through a filter candle. We are inclined to believe that the method of sterilisation of the carbohydrate is important, and that after sterilisation by steaming there is likely to be a bigger disappearance of a sugar in culture than when the unheated sugar is used. The large breakdown of glucose observed in our earlier work may have been due to the fact that the medium was sterilised by heat. This idea is born out to some extent by an experiment with adult rat kidney tissue, in which the disappearance of steamed and unsteamed glucose in the presence of the tissue was estimated. It was found that there was a larger disappearance of the sugar which had been steamed. The sugar may or may not have been broken down by the tissue. Two or three years ago, one of us (B.E.H.) attempted in collaboration with A. Pirie to repeat the work of Borsook and Wasteneys [1925] in which they showed that glucose in the presence of an amino-acid and phosphate could reduce methylene blue under anaerobic conditions. We found that we could not repeat this unless we used, as did Borsook and Wasteneys themselves, glucose which had been sterilised by heating. With fresh glucose no reduction of the methylene blue was obtained. In these tissue culture experiments, therefore, it seemed safer to avoid complications by using filtered instead of steamed solutions.

Other details of technique will be found in earlier papers.

Growth[1] *of tissue.*

In most series one or more small control dishes were included, containing small amounts of tissue growing in the same medium as that used in the experimental flasks. This tissue could be stained and used for microscopical purposes. We have not adopted any accurate method for estimating growth, but the presence of fructose, galactose, and even xylose seemed to have an unmistakably favourable effect upon the growth of the tissues. This was particularly noticeable when the growth without added sugar was poor. From this it seemed almost certain that each of these sugars was utilised by the tissue, and we found later that definite amounts of the carbohydrate disappeared during the course of the experiment.

Effect on production of ammonia and urea.

Fructose behaved like glucose in that it inhibited either partially or completely the production of ammonia and urea by growing tissue in spite of the increased growth promoted by the sugar. In the presence either of glucose [Watchorn and Holmes, 1927] or of fructose (Table I) there was sometimes an actual disappearance of the urea already present, and as this did not appear as extra ammonia it seems possible that it was used by the growing tissue.

Table I. *Effect of fructose upon ammonia and urea production.*

	Non-growing tissue		Growing tissue		Non-growing tissue + fructose		Growing tissue + fructose		Increase in NH_3-N + urea-N		
Exp. no.	NH_3-N (mg.)	NH_3-N + urea-N (mg.)	NH_3-N (mg.)	NH_3-N + urea-N (mg.)	NH_3-N (mg.)	NH_3-N + urea-N (mg.)	NH_3-N (mg.)	NH_3-N + urea-N (mg.)	Without fructose (mg.)	With fructose (mg.)	Effect of fructose
80	0·034	0·051	*0·040 0·044	0·070 0·065	0·030	0·058	0·030 0·033	0·066 0·061	0·019 0·014	0·008 Nil	Inhibition
81	—	0·056	—	0·118 0·092	—	0·055	0·036	0·081	0·062 0·036	0·026	Partial inhibition
82	0·031	0·060	0·038	0·071	0·030	0·062	0·030	0·055	0·011	Nil	Inhibition
84	0·040	0·097	0·059 0·050	0·163 0·117	0·040	0·081	0·057 0·040	0·077 0·088	0·066 0·020	0·016 0·007	Partial inhibition
86	0·043	0·066	0·060	0·105 0·072	0·039	0·067	0·043	0·054 0·056	0·039 Nil	Nil Nil	Inhibition (possible urea utilisation)

* Where two sets of figures are given, these refer to two separate preparations in the same experimental series, the control therefore being the same for both.

Quite unlike the results obtained with fructose and xylose those with galactose were markedly irregular. This irregularity persisted, although the experiments were repeated a great many times. Sometimes the production of ammonia and urea was inhibited, sometimes it was unaffected or even slightly increased (the increase probably being due to the relatively greater growth). Perhaps there is more than one path of galactose breakdown possible in the cultures, and the effect upon the nitrogen metabolism depends upon the path taken. It is certain, however, that the presence of galactose could

[1] By "growth" we mean increase of area, which is due to wandering of cells as well as to actual cell division.

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82	0·031	0·060	0·038	0·071	0·030	0·062	0·030	0·055	0·011	Nil	Inhibition
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at times have an inhibitory effect upon the formation of ammonia and urea, and when concentrations of sugar as high as 0·2 % or more were used, this inhibition was likely to be found in the majority of cases. Some results are given in Table II.

Table II. *Effect of galactose upon ammonia and urea production.*

	Non-growing tissue		Growing tissue		Non-growing tissue + galactose		Growing tissue + galactose		Increase in NH_3-N + urea-N		
Exp. no.	NH_3-N (mg.)	NH_3-N + urea-N (mg.)	NH_3-N (mg.)	NH_3-N + urea-N (mg.)	NH_3-N (mg.)	NH_3-N + urea-N (mg.)	NH_3-N (mg.)	NH_3-N + urea-N (mg.)	Without galactose (mg.)	With galactose (mg.)	Effect of galactose
76	0·030	0·045	0·024	0·039	0·029	0·043	0·037	0·072	Nil	0·029	Increase
	0·030	0·045			0·026	0·045	0·025	0·036		Nil	No effect
79	0·032	0·063	0·055	0·073	0·032	0·063	0·039	0·073	0·010	0·010	No inhibition
			0·055	0·088			0·025	0·056	0·025	Nil	Inhibition
			0·045	0·074			0·037	0·056	0·011	Nil	Inhibition
90	0·057	0·090	0·089	0·136	0·062	0·116	0·062	0·118	0·046	Nil	Inhibition
91	—	0·083	—	0·084	—	0·085	—	0·112	Nil	0·027	Increase
93	0·074	0·135	0·098	0·172	0·055	0·103	0·095	0·115	0·037	0·052	Increase
98	0·029	0·083	0·041	0·099	0·029	0·083	0·036	0·090	0·016	0·007	Partial inhibition
					Higher concentrations of galactose (approx. 0·2 %).						
118	—	0·108	—	0·123	—	0·108	—	0·103	0·015	Nil	Inhibition
								0·033		0·025	Small increase
119	—	0·088	—	0·136	—	0·097	—	0·090	0·048	Nil	Inhibition

In no case have we found any inhibition of ammonia or urea formation by xylose, although there seems to be very little doubt that the tissue can cause this sugar to disappear from the medium. Xylose therefore is an interesting case of a carbohydrate which is in some way utilised without affecting the nitrogen metabolism of the tissue as measured by the production of these substances (Table III).

Table III. *Effect of xylose (0·2 %) upon ammonia and urea production.*

	Non-growing tissue		Growing tissue		Non-growing tissue + xylose		Growing tissue + xylose		Increase in NH_3-N + urea-N		
Exp. no.	NH_3-N (mg.)	NH_3-N + urea-N (mg.)	NH_3-N (mg.)	NH_3-N + urea-N (mg.)	NH_3-N (mg.)	NH_3-N + urea-N (mg.)	NH_3-N (mg.)	NH_3-N + urea-N (mg.)	Without xylose (mg.)	With xylose (mg.)	Effect of xylose
99	0·032	0·091	0·049	0·119	0·040	0·097	0·052	0·121	0·028	0·024	No inhibition
							0·055	0·121		0·024	
101	0·026	0·056	0·030	0·078	0·036	0·066	0·040	0·100	0·022	0·034	Slight increase
			0·035	0·082					0·026		
102	0·024	0·048	0·031	0·067	0·030	0·063	0·045	0·079	0·019	0·016	No inhibition
120	—	0·070	—	0·113	—	0·080	—	0·129	0·043	0·049	No inhibition
121	—	0·102	—	0·135	—	0·083	—	0·114	0·033	0·041	No inhibition

Disappearance of the sugars from the medium.

Apart from the added sugar the medium contained very little reducing substance. This is to be expected, since it was made from chopped embryo tissue which had already been once washed with Ringer's solution, and was finally diluted with twice its volume of Ringer's solution. The amount of reducing substance was about 0·1 mg. to 0·15 mg. in each culture. This is

unimportant when compared with the amount of sugar added (2·0 mg. approximately in each culture). We did not find that it changed appreciably during the experiment, and in any case it was not sufficient to account for the changes observed. We therefore ignored the reducing substances already present, and estimated the added sugar as reducing substance. The small amount of protein present was removed with colloidal ferric hydroxide, and the sugar estimated by the method of Hagedorn and Jensen. In order to obtain larger percentage differences we have made the final concentration 0·1 % instead of the 0·2 % used in most of the other experiments.

There seemed to be very little doubt that all the sugars added, fructose, galactose and xylose, could be utilised by the tissue, and it will be seen that the non-growing generally used less than the growing tissue. The control was a culture which had been kept in the ice-chest throughout the experimental period (Tables IV, V, VI).

Table IV. *Utilisation of fructose (estimated as glucose).*

Exp. no.	Control at 0° (mg.)	Non-growing tissue (mg.)	Growing tissue (mg.)	Control – growing tissue (mg.)
114	1·61	(1) 1·25 (2) 1·23	(1) 1·23 (2) 1·20	(1) 0·38 (2) 0·41
110	1·38	1·22	1·10	0·28
109	3·52*	(1) 3·20	(1) 3·23	(1) 0·29

* Very little tissue used.

It is interesting to find a small but definite disappearance of fructose, as it has been suggested [Campbell and Markowitz, 1927] that fructose is not utilised except by the liver.

Table V. *Utilisation of galactose (estimated as glucose).*

Exp. no.	Control at 0° (mg.)	Non-growing tissue (mg.)	Growing tissue (mg.)	Control – growing tissue (mg.)
115	1·39	1·02	(1) 1·06 (2) 0·93	(1) 0·33 (2) 0·46
116	1·35	0·99	0·67	0·68*

* Grew for 3 days.

It has been shown that galactose can give rise to lactic acid [Wierzuchowski and Laniewski, 1931] and can raise the respiratory quotient and the heat production of dogs [Wierzuchowski, 1931], while Sherif and E. G. Holmes [1930] have observed an increased oxygen uptake with mammalian nerve in the presence of galactose. It is perhaps worth noting that on one occasion the rise in blood- and urine-lactic acid found by Wierzuchowski and Laniewski was much greater than in the other two experiments; some such irregularity may explain the lack of consistency in the effect of galactose on nitrogen metabolism in our experiments.

Table VI. *Utilisation of xylose (estimated as glucose).*

Exp. no.	Control at 0° (mg.)	Non-growing tissue (mg.)	Growing tissue (mg.)	Control – growing tissue (mg.)
113	2·03	—	1·70	0·33
106	1·81*	1·77	1·73	0·08
122	2·00†	1·60	1·46	0·54
108	1·99	1·77	1·64	0·35

* Very little tissue used.

† In this experiment the sugar was estimated by McCance's [1926] method for pentoses, and we are glad to have this opportunity of thanking him for help.

McCance and Madders [1930] showed that xylose was retained in the tissues after injection or absorption from the intestine, and Russell [1923] showed that pentoses were oxidised by tumour tissues, but that normal nerve tissues, including kidney, cannot oxidise them. The only exception was liver, which oxidised arabinose. He did not however use any embryonic tissue, and we think that the fact that we used embryonic tissue in an active condition influenced the results with xylose and with the other sugars.

Summary.

1. The tissue culture method has been used to test on embryo kidney tissue the "protein sparing" action of fructose, galactose and xylose.

2. Each of these sugars tends to increase the growth of the tissue and each of them is utilised by the tissue, but while fructose constantly inhibits the production of ammonia and urea by the growing cells, galactose does so only irregularly and xylose not at all.

We are indebted to the Medical Research Council for personal grants.

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XCVI. THE EFFECT OF NARCOTICS ON SOME DEHYDROGENASES.

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According to the well-known work of Warburg [1921] the inhibiting effect of narcotics on cell respiration, on fermentation by yeast and on oxidation of amino-acids on a charcoal surface is due to the preferential adsorption of these substances on the particular surfaces, and the reaction velocity is inhibited owing to the displacement of the reacting substances in question by the narcotics. That the narcotic action increases with the increase in the molecular weight in a homologous series, coupled with the fact that the surface activity of these substances increases also in the same order, is a strong support for the theory of Warburg. Nothing is known, however, about the particular constituents of the cell on which this displacement of adsorption takes place, though a good deal of emphasis has been laid on the rôle of lipoids in the phenomenon of narcosis [Loewe, 1913]. Henderson [1930] in his recent review of the theories of narcosis finds considerable difficulties in deciding on the substrate on which narcotics act in the known reversible manner. This question of the effect of narcotics on some particular constituents of the cells is naturally interesting and requires elucidation before the narcosis of cell respiration can be properly understood.

In a study of the cytochrome-indophenol oxidase system of cells, Keilin [1929] has put forward the view that cyanide inhibits the oxidation of cytochrome by the indophenol oxidase system. Narcotics, however, have little or no effect on the activity of the oxidase-cytochrome system. That is, they do not affect the oxidation of reduced cytochrome, but they inhibit the activity of dehydrogenases and hence the reduction of oxidised cytochrome. The inhibiting effect of narcotics on cell respiration is thus due to their inhibiting action on dehydrogenases in the cells.

The present paper contains a study of the effect of some narcotics on three dehydrogenase systems, namely xanthine-hypoxanthine dehydrogenase (xanthine oxidase), aldehyde dehydrogenase (Schardinger enzyme) and succinic dehydrogenase. Incidentally some experiments have been made on the question of the identity of xanthine oxidase with Schardinger enzyme.

Experimental.

The xanthine oxidase and Schardinger enzyme were prepared in the same sample in the form of the caseinogen preparation of Dixon and Thurlow

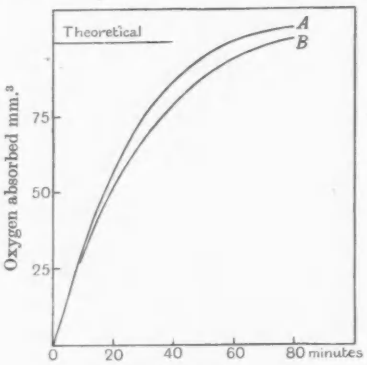

Fig. 1.

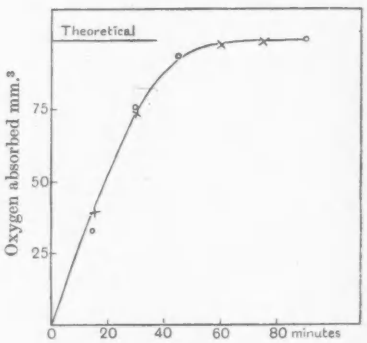

Fig. 2.

Fig. 1. Effect of ethylurethane on xanthine oxidase-hypoxanthine system.
A. Control. B. Urethane 5 %.

Fig. 2. Effect of phenylurethane on xanthine oxidase system.
o—o—o Denotes control. ×—×—× Denotes urethane-saturated.

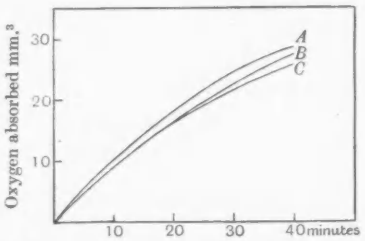

Fig. 3. Effect of narcotics on Schardinger enzyme.
A. Phenylurea 0·02 *M*. B. Control and valeronitrile 0·11 *M*.
C. Ethylurethane 5 %.

The Barcroft cups in all these cases (Figs. 1, 2 and 3) were made up as follows: 0·5 cc. of 20 % caseinogen solution and 1·2 cc. phosphate buffer p_H 7·6 were placed in both the cups. In the control, 0·3 cc. of 0·2 % hypoxanthine + 1 cc. of water was added in the right hand cup, whilst the left hand cup received 1·3 cc. water. The hypoxanthine was contained in a small Keilin tube which was tipped into the cup after temperature equilibration in the usual way and this was taken as the zero time. In the case of Schardinger enzyme, 0·5 cc. of 0·25 % piperonal was added in place of hypoxanthine, the quantity of water being reduced to 1·1 cc. When narcotics were added, the amount of water was reduced proportionately, the total volume being kept constant at 3 cc. in both the cups. The narcotic was added in both the cups, and in the case of phenylurethane an excess was present.

[1924, 1] from highly active milk. The succinic dehydrogenase was prepared from washed sheep's heart muscle according to the method of Keilin [1929].

The experiments were made both by the methylene blue technique (anaerobically) and by the oxygen-uptake method in a Barcroft micro-respirometer. The main results are summarised below. All the experiments were made at 37°.

(1) With xanthine oxidase using hypoxanthine as substrate, ethylurethane 5 %, phenylurethane, saturated solution, diethylurea 5 % and phenylurea 0·3 % had very little effect on the velocity of oxygen-uptake (Figs. 1 and 2).

(2) With Schardinger enzyme, using piperonal as substrate, ethylurethane 5 %, phenylurea 0·02 *M*, and valeronitrile 0·11 *M* had very little effect on the initial velocity of oxygen-uptake (Fig. 3).

(3) In the anaerobic reduction of methylene blue in presence of xanthine oxidase and Schardinger enzyme, similar results were obtained. The amount of methylene blue used was 1 cc. of 1:5000 solution in 5 cc. total volume.

(4) Narcotics had a strong inhibiting action on the succinic dehydrogenase system, the results of the oxygen-uptake and methylene blue reduction methods agreeing with each other. This action was found to be (in the case of ethylurethane which was the only narcotic examined) completely reversible. As the data are interesting, these results are given in some detail.

Effect of narcotics on succinic dehydrogenase.

The narcotics used in this investigation were ethylurethane, phenylurethane, diethylurea, phenylurea, propionitrile, valeronitrile and vanillin. The dehydrogenase was a washed muscle preparation containing approximately 100 mg. per 1 cc. of the suspension. The Barcroft cups were made up as follows: 0·5 cc. muscle preparation, 1 cc. phosphate buffer p_H 7·6, 1 cc. narcotic of known concentration. The right-hand cup contained in addition 0·5 cc. *M*/10 sodium succinate, whilst the same amount of water was added to the left-hand cup. In the control experiments, of course, no narcotic was added. The total volume

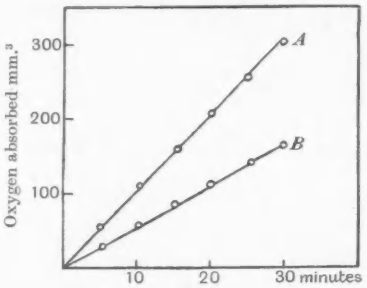

Fig. 4.

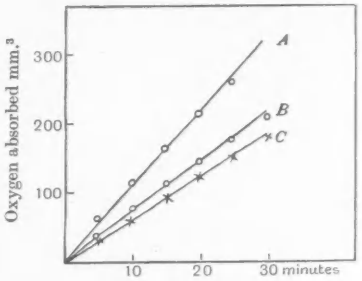

Fig. 5.

Fig. 4. Effect of diethylurea on the succinic dehydrogenase system.
A. Control. B. Diethylurea 0·66 *M*.

Fig. 5. Effect of phenylurea and vanillin on the succinic dehydrogenase system.
A. Control. B. Phenylurea 0·02 *M*. C. Vanillin 0·011 *M*.

in every cup was 3 cc. and the temperature of the bath 37°. In Figs. 4 to 7 the results are graphically shown. It will be observed that with all these narcotics the activity of the oxygen-absorbing system is greatly reduced.

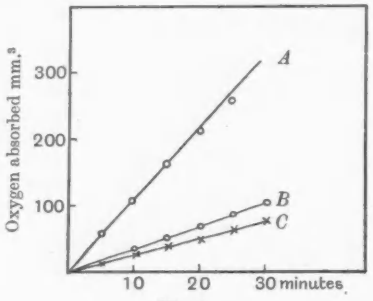

Fig. 6.

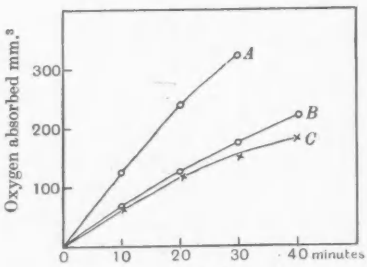

Fig. 7.

Fig. 6. Effect of nitriles on the succinic dehydrogenase system.
A. Control. B. Valeronitrile 0·11 *M*. C. Propionitrile 0·66 *M*.

Fig. 7. Effect of urethanes on the succinic dehydrogenase system.
A. Control. B. Phenylurethane 0·003 *M*. C. Ethylurethane 0·66 *M*.

The majority of the narcotic concentrations were chosen in such a way (by preliminary tests) that the inhibition fell to about 50 % of the control. In other cases this value has been extrapolated. The following table summarises the results.

Table I.

	Concentration of narcotic which inhibits about 50 % (*M*)		
Narcotic	Oxygen-uptake in presence of succinic dehydrogenase	Respiration of nucleated erythrocytes—Warburg	Anaerobic reduction of methylene blue by succinic dehydrogenase
Ethylurethane	0·65	0·33	0·6
Phenylurethane	0·003	0·003	0·002
Diethylurea	0·35	0·52	0·2
Phenylurea	0·028	0·018	0·028
Propionitrile	0·48	0·36	—
Valeronitrile	0·08	0·06	—
Vanillin	0·011	0·02	0·022

The results obtained in this work are compared with those of Warburg on respiration by avian blood corpuscles. The last column contains the results obtained by the methylene blue technique. Since it will be interesting to compare the results of these methylene blue reduction experiments with the results obtained in oxygen absorption experiments, some of them are given here. It will be observed that the effects of narcotics in the case of the succinic dehydrogenase system are really on the hydrogen-activating mechanism of the system. Figs. 8–12 illustrate the main results obtained. Each Thunberg tube contained 1 cc. phosphate buffer of p_H 7·6, 0·5 cc. methylene blue 1:5000, 0·5 cc. muscle preparation, and 0·5 cc. sodium succinate *M*/10 unless

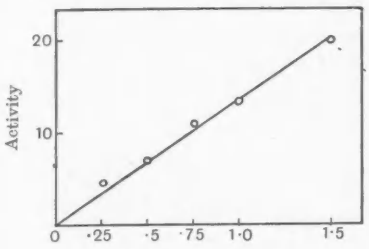

Fig. 8.

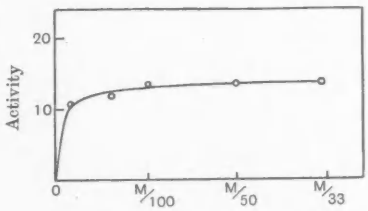

Fig. 9.

Fig. 8. Effect of varying enzyme concentration on the succinic dehydrogenase system.
Fig. 9. Effect of varying substrate concentration on the succinic dehydrogenase system.

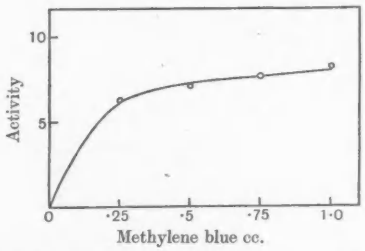

Fig. 10. Effect of varying methylene blue concentration on the succinic dehydrogenase system. The curve shows the activity per unit quantity of methylene blue. The original concentration of methylene blue is 1 in 5000.

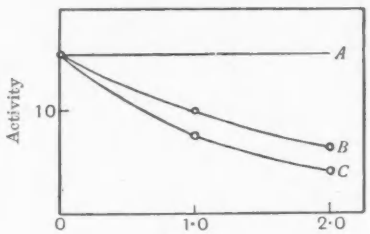

Fig. 11.

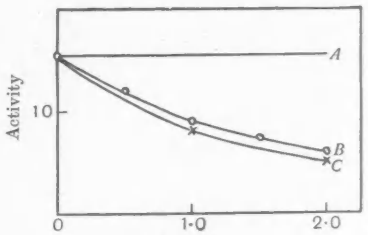

Fig. 12.

Fig. 11. Effect of urea on the succinic dehydrogenase system.
A. Activity without narcotic. B. Phenylurea 0·06 *M* solution.
C. Diethylurea 1·0 *M* solution.

Fig. 12. Effect of urethanes on the succinic dehydrogenase system.
A. Activity without narcotic. B. Ethylurethane 17·8 %.
C. Phenylurethane 0·05 %.

otherwise stated, the total volume being 5 cc., made up either by addition of water or narcotic. The activity is the reciprocal of the time of reduction of methylene blue in minutes multiplied by 100. The data include the effect of varying enzyme concentration, the effect of different methylene blue concentrations, the effect of different substrate concentrations and the effect of different narcotics of different concentrations. The data given in the last column of Table I have been obtained from the experimental curves. It will be observed that the velocity of the reduction of methylene blue (activity) is directly proportional to the enzyme concentration, that the rate of methylene blue reduction at constant enzyme concentration depends on the concentration of methylene blue below a certain concentration, and that the rate of reduction of methylene blue is nearly constant within the range of substrate concentration studied in this paper [compare Ahlgren, 1926]. It will also be seen from Table I that in every case the higher homologue has a greater inhibiting (narcotic) action, a fact also observed by Batelli and Stern [1913, 1] but criticised by Vernon [1914], and the magnitudes of the individual values are also often not far from the values obtained by Warburg in his experiments on respiration by blood cells.

It will thus be observed from the above results that the muscle system containing succinic dehydrogenase and indophenol oxidase gives results in presence of narcotics entirely in accordance with the theory of Keilin. It is therefore interesting to enquire why narcotics fail to show any effect on xanthine oxidase or Schardinger enzyme. Curiously enough, both these systems are also not sensitive to cyanide, whereas oxidation of succinate in presence of the muscle system is sensitive to cyanide. This is no doubt due to the fact that cyanide inhibits the indophenol oxidase system which is absent from the xanthine or Schardinger enzyme. It is to be noted at this place that nitriles (propionitrile, valeronitrile) had no effect on the reduction of methylene blue by succinic dehydrogenase, though they inhibited the oxygen absorption. This is to be explained by the fact that the .CN group does not affect the hydrogen-activating system. Regarding the difference in the behaviour of these two types of dehydrogenases in presence of narcotics nothing definite can be said at present, but some related facts may be stated here. Batelli and Stern [1913, 2] showed that there is some connection between the ability of narcotics to coagulate nucleoproteins and to inhibit the action of succinic dehydrogenase. The muscle preparation used in this work is a coarse suspension which easily settles down in presence of narcotics. The caseinogen preparation of xanthine oxidase or Schardinger enzyme is, however, more soluble and gives a slightly opalescent solution which is not precipitated in presence of narcotics of the concentration used in this investigation. It was found, however, in several instances that by increasing the concentration of the narcotic to a high value (for instance, ethylurethane 14 %), some diminution in the velocity of oxygen-uptake in presence of xanthine oxidase could be observed. This effect may be due to a real coagula-

tion effect. As however such high concentrations are not to be considered as narcotic concentrations, these results were not followed up by any further work. Mention may also be made here of the interesting attempt of Bernard [1885], who tried to classify narcotisable and non-narcotisable ferments on the basis of their being associated either with living protoplasmic mass or with non-living and non-protoplasmic mass.

The identity of xanthine oxidase with Schardinger enzyme.

Warburg in his experiments used vanillin as a narcotic, and it has been found by me in this paper to have a narcotic action on succinic dehydrogenase. Since vanillin is an aldehyde, it could not be used as a narcotic as such with the caseinogen preparation of Dixon and Thurlow, as this preparation catalyses the oxidation of both hypoxanthine and aldehydes. Some preliminary experiments on the oxidation of hypoxanthine in presence of the caseinogen preparation, however, showed that the presence of vanillin affects the rate of hypoxanthine oxidation. It has previously been observed by Dixon and Thurlow [1924, 2] that uric acid inhibits the oxidation of aldehydes in presence of Schardinger enzyme and they advance this as a strong point in favour of the view that xanthine oxidase and Schardinger enzyme are identical. This view is also strongly supported by the high degree of specificity of the enzymes found by Coombs [1927]. The result obtained by me points to the fact that in presence of vanillin the two substrates are competing with each other, producing a competitive inhibition. The competition is so great that in experiments in which a saturated solution of vanillin was placed in both the cups of a Barcroft apparatus and hypoxanthine only in the right cup, no movement of the liquid in the manometer took place in half an hour. This showed that the oxygen-uptake in the two cups was balanced and the presence of a saturated solution of vanillin had completely excluded the hypoxanthine from the enzyme surface. Since Wieland and Macrae [1930] consider that they have observed a summation effect with two substrates (hypoxanthine and aldehyde), it was thought worth while to obtain some comparative data on this point. The experiments have shown that with hypoxanthine on one hand and vanillin (*m*-methoxy-*p*-hydroxybenzaldehyde) and piperonal (3:4-methylenedioxybenzaldehyde) on the other hand, no additive effect was observed. In every case the combined velocity of oxygen-uptake was midway between the velocities of oxygen-uptake of the separate systems. Figs. 13 and 14 show the results. The concentration of vanillin used was 0·5 % and of piperonal 0·25 %, other conditions being the same as described before. It will be seen that as the aldehyde concentration increases the total oxygen-uptake falls, though, had there been two different enzymes, the oxygen-uptake would have been greater than when hypoxanthine was present alone. The decrease in the oxygen-uptake is due to the fact that the aldehyde, which has a much lower rate of oxygen-uptake, displaces a portion of the hypoxanthine and hence lowers the rate of oxygen-uptake of the whole system. This result

points very strongly to the conclusion that the two dehydrogenases are identical.

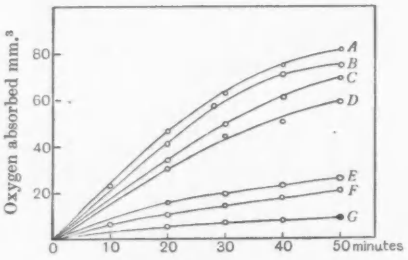

Fig. 13.

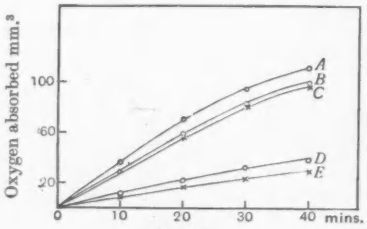

Fig. 14.

Fig. 13. Effect of vanillin on hypoxanthine oxidation.

A. 0·3 cc. hypoxanthine : no vanillin.
B. 0·3 cc. hypoxanthine + 0·2 cc. vanillin.
C. 0·3 cc. hypoxanthine + 0·5 cc. vanillin.
D. 0·3 cc. hypoxanthine + 1·0 cc. vanillin.
E. 1·0 cc. vanillin : no hypoxanthine.
F. 0·5 cc. vanillin : no hypoxanthine.
G. 0·2 cc. vanillin : no hypoxanthine.

Fig. 14. Effect of piperonal on hypoxanthine oxidation.

A. 0·3 cc. hypoxanthine : no piperonal.
B. 0·3 cc. hypoxanthine + 0·2 cc. piperonal
C. 0·3 cc. hypoxanthine + 0·5 cc. piperonal.
D. 0·5 cc. piperonal only.
E. 0·2 cc. piperonal only.

In these experiments (Figs. 13 and 14) the concentration of vanillin was 0·5 % and of piperonal 0·25 %, both prepared in phosphate buffer of p_H 7·6. The Barcroft cups were made up as follows. The right-hand cup usually received a constant amount of 0·3 cc. hypoxanthine 0·2 % solution (unless otherwise stated) and varying amount of either vanillin or piperonal. 0·5 cc. of the 20 % caseinogen solution was kept in the small Keilin tubes and was tipped into both the cups after temperature equilibration, and this was taken as the zero time. The total volume was adjusted at the beginning to 3 cc. by the addition of phosphate buffer in both the cups. Control experiments were also made without any hypoxanthine.

Summary.

1. Narcotics have no effect on xanthine oxidase or Schardinger enzyme (caseinogen preparation) in ordinary concentrations, either as regards oxygen absorption or anaerobic reduction of methylene blue.

2. Narcotics have a great inhibiting action on the succinic dehydrogenase system, the effect increasing with higher homologues of the same series. The degrees of inhibition of oxygen-uptake and methylene blue reduction agree with each other in the majority of cases. Nitriles have, however, no effect on methylene blue reduction, though they inhibit the oxygen-uptake strongly.

3. A competitive inhibition of hypoxanthine oxidation is observed in presence of vanillin and piperonal. This is considered to be a strong point in favour of the view that xanthine oxidase and Schardinger enzyme are identical.

In conclusion I desire to express my thanks to Dr D. Keilin and Dr Malcolm Dixon for their helpful advice and criticism and to Sir F. G. Hopkins for his kind interest in this work.

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XCVII. OXIDATION-REDUCTION POTENTIALS OF HAEMOLYTIC STREPTOCOCCI.

II. EFFECT OF CATALASE.

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Peroxide formation has a marked effect on the electrode potential behaviour of bacterial cultures and it has been shown in previous communications that the course of development of reducing and oxidising conditions in cultures of the peroxide-forming organisms haemolytic streptococci [Hewitt, 1930, 1] and pneumococci [1930, 4] differs characteristically from that in cultures of the catalase-containing bacteria *C. diphtheriae* [1930, 2] and staphylococci [1930, 3]. Certain of the characteristics in the behaviour of pneumococcus disappear when peroxide accumulation is prevented by addition of catalase to the cultures [1931, 1]. Nevertheless differences still remain between the reducing activities of pneumococcus cultures containing catalase on the one hand and, say, staphylococcus cultures on the other.

Hydrogen peroxide is a common by-product in biological oxidation-reductions and has a very definite effect on electrode potentials. It is clear therefore that the presence or absence of catalase (or other peroxide-decomposing system) must have a pronounced effect on the potentials developed in bacterial cultures. It is important, however, to bear in mind that the presence or absence of other enzymes concerned in biological oxidation-reduction reactions must also have marked effects. McLeod [1930] has suggested that the phenomena of anaerobiosis may be ascribed to peroxide formation, but it is probable that other factors are involved [Hewitt, 1931, 2]. It is of interest, therefore, to determine the differences in the oxidation-reduction potentials developed in cultures of different organisms when peroxide formation is eliminated by the addition of catalase, and this short communication is an account of the effect of catalase on the oxidation-reduction potentials of cultures of haemolytic streptococci.

Methods.

Electrode potentials of cultures were measured by means of the apparatus and methods previously described [1930, 1; 1931, 2]. The glossy variant of Aronson-Schnitzer strain of haemolytic streptococci [Todd, 1930, 1] was used and the inoculum of 0·1 cc. of a 24 hour broth culture was made into 8 cc. of medium in each experiment. Cultures were incubated at 37° and readings of the potential were made every 30 minutes during the first 12 hours' incubation.

The catalase preparation used was prepared from pig's liver by the method of Batelli and Stern [1904]. The liver was extracted with water, the catalase was precipitated by addition of alcohol, filtered off, redissolved in water, re-precipitated by alcohol, filtered off and dried *in vacuo*. Catalase broth was prepared by suspending the highly active catalase preparation in broth in the required concentration, and after standing for an hour the broth was filtered through a sterile Seitz filter. The catalase preparation did not dissolve completely, but the filtered broth decomposed hydrogen peroxide vigorously even after standing at room temperature for 2 months. 1 % and 0·2 % catalase broth were used, that is 1·0 or 0·2 g. of the catalase powder suspended in 100 cc. of broth.

Results.

No differences were detected between the electrode potential of plain broth and that of broth to which the catalase preparation had been added 24 hours previously.

Stationary aerobic cultures (Fig. 1). In plain peptone infusion broth cultures

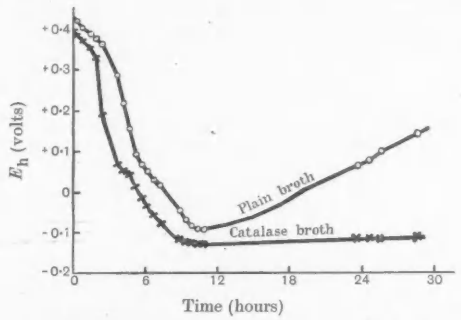

Fig. 1. Stationary aerobic cultures (peptone-infusion broth).

the potential fell to its minimum value of about − 0·1 v. in 12 hours. The potential had risen to + 0·07 v. in 24 hours and to + 0·16 v. in 30 hours. This rapid rise in potential after the logarithmic phase of growth is characteristic of peroxide-forming bacteria. In broth containing 0·2 % of the liver catalase preparation the potential fell to a slightly lower level than that of the plain broth culture and after 30 hours' incubation was still below − 0·11 v. The catalase had thus inhibited the usual rise in potential after the logarithmic phase of growth and had brought the behaviour of haemolytic streptococci more into line with that of staphylococci or *C. diphtheriae*. The behaviour of pneumococci in the presence of catalase was similar. The disappearance of reducing conditions after the logarithmic phase of growth is evidently associated with peroxide formation, although no peroxide can be detected in aerobic cultures under these conditions.

The presence of high concentrations of the catalase preparation in broth

(*e.g.* 1 %) produced a definite poising effect on the potentials, probably owing to the presence of extraneous substances such as haematin derivatives.

Aerated cultures (Fig. 2). Cultures were aerated in three-limbed cells by

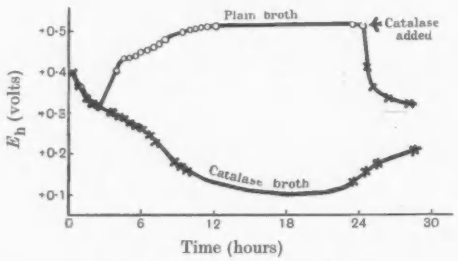

Fig. 2. Aerated digest-broth cultures.

the method previously described [1930, 1]. In the plain tryptic digest broth cultures the potential fell to a minimum level of + 0·31 v. in 2·5 hours and then rose rapidly to + 0·5 v., at which level peroxide may be detected in the culture. In the presence of 0·2 % catalase however the potential fell to a level of about + 0·1 v. and after 30 hours' incubation had not risen above + 0·2 v. The addition of catalase, therefore, had prevented peroxide formation, with the result that the potential fell to a much lower level and did not rise to the high level corresponding to peroxide formation. A similar effect was seen in the case of pneumococci [1931, 1].

As a matter of interest a small quantity of the catalase preparation was added to the aerated plain broth culture after 25 hours' incubation. There was evident effervescence of oxygen as the peroxide present was decomposed. Despite this vigorous oxygenation the potential fell rapidly. The fall was 0·1 v. within 10 minutes after the addition of the catalase, and the total fall in 4 hours was 0·2 v. Two conclusions may be drawn from this experiment; first, it is evident that peroxide formation accounted for the high potential since the addition of catalase resulted in an immediate fall; second, the relatively inert nature of molecular oxygen is demonstrated. Although hydrogen peroxide may be regarded as a reduction product of oxygen, yet peroxide has a much higher oxidising potential. The tendency to part with molecular oxygen evidently does not account for the oxidising effect of hydrogen peroxide.

Discussion.

Bacterial peroxide formation is of considerable importance since it has been suggested as a criterion for the classification of bacteria, as an explanation of the behaviour of obligate anaerobes [McLeod, 1930] and as a factor in bacterial variation [Todd, 1930, 1, 2, 3]. The glossy variant of haemolytic streptococci is less sensitive to peroxide than the matt variants, hence there is a natural selection of glossy variants when peroxide is formed. When,

however, peroxide formation is inhibited by the presence of catalase the degradation of matt to glossy variants does not occur [Todd, 1930, 1]. The glossy variants are avirulent, whilst the matt form may be virulent or attenuated, and the virulence is maintained on long continued artificial cultivation only when the oxygen supply is abundant and peroxide accumulation in the culture is avoided [Todd, 1930, 2]. The effect of catalase on the oxidation-reduction conditions developed in cultures of haemolytic streptococci is, therefore, of more than academic interest.

Addition of catalase to the cultures inhibits peroxide formation by haemolytic streptococci and results in corresponding modifications of the electrode potential-time curves. In aerobic catalase broth cultures the potential remains at a low level long after the cessation of active proliferation and does not rise as in plain broth cultures. In aerated cultures the potential falls to a lower level in the presence of catalase and the usual high level corresponding to peroxide formation is not reached. The effect of catalase on cultures of haemolytic streptococci is therefore closely similar to that observed with pneumococci [1931, 1].

When a small quantity of the catalase preparation was added to an aerated culture of haemolytic streptococci in which peroxide had been formed the potential immediately fell very rapidly although oxygen was visibly liberated throughout the culture. This presents confirmatory evidence that

(1) the effect of the enzyme preparation on the potentials of growing cultures was due to catalase activity and not to extraneous factors;

(2) bacterial peroxide is closely similar to, if not identical with, hydrogen peroxide;

(3) peroxide has a much greater effect on the potential than molecular oxygen.

In many respects, cultures of haemolytic streptococci to which catalase has been added behave qualitatively like those organisms which produce their own catalase. Quantitative differences, however, still remain. Thus the potential-time curves of aerated cultures of pneumococci and haemolytic streptococci are entirely different from those of staphylococci and *C. diphtheriae*. When catalase is added to cultures of pneumococci and haemolytic streptococci the potential-time curves are now roughly similar in form to those of the catalase-containing bacteria; but the minimum values of potential reached vary widely, being approximately: pneumococci + 0·15 v., haemolytic streptococci + 0·05 v., staphylococci − 0·05 v. and *C. diphtheriae* − 0·15 v. These figures are only roughly comparable since they were obtained on different occasions with different samples of broth *etc.* They are sufficient, however, to indicate the great differences in reducing activity of these different bacteria. In aerobic cultures not subjected to special aeration the differences are much smaller, the minimum values being approximately pneumococci − 0·16 v., haemolytic streptococci − 0·17 v., staphylococci − 0·18 v. and *C. diphtheriae* − 0·20 v.

Thus, although the formation or non-formation of peroxide may be a valuable criterion for the classification of bacteria and may have important effects on bacterial variation, it should not be regarded as the only determining factor in bacterial behaviour.

SUMMARY.

1. Addition of catalase inhibits peroxide formation in cultures of haemolytic streptococci and the effect is closely similar to that observed with pneumococci.

2. Reducing conditions are maintained after the logarithmic phase of growth when catalase has been added to the culture.

3. The electrode potential falls to a lower level in aerated cultures when catalase is added, and the high level corresponding to peroxide formation is not reached.

4. Addition of catalase to a culture containing peroxide causes an immediate and rapid fall in potential despite the resulting vigorous oxygenation of the culture.

The author acknowledges his indebtedness to Dr R. G. White and Dr E. W. Todd for their continued help and encouragement.

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XCVIII. A METHOD FOR THE DETERMINATION OF SMALL QUANTITIES OF MIXED REDUCING SUGARS AND ITS APPLICATION TO THE ESTIMATION OF THE PRODUCTS OF HYDROLYSIS OF STARCH BY TAKA-DIASTASE.

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(*Received May 1st, 1931.*)

Preliminary investigations on the carbohydrates in the developing apple have shown that the concentration of sugars in the fruit is less than 1 % during the first weeks of growth, and further, that the maximum starch concentration reached at any time is only about 2 %. Estimation of these carbohydrates by the usual methods would require the collection of a very large number of apples, especially in the early stages of development, when the fruit is small, with the resultant risk that the crop on any tree would be so seriously depleted that the development of the remaining fruit might be abnormal.

Since starch appears to be most satisfactorily estimated by determination of the glucose and maltose produced by hydrolysis with taka-diastase, a method for the estimation of small quantities of reducing sugars has been investigated. With such a method, determinations of starch could be carried out during the development of the apple, and furthermore, a delicate method of estimation of reducing sugar would enable the size of the samples required for sugar determination on the young and very small fruits to be kept within reasonable limits.

Estimation of small amounts of reducing sugars.

In order to determine two mixed reducing sugars it is necessary to employ two methods of estimation, and to solve the pair of simultaneous equations obtained from the results of the determinations.

Hagedorn and Jensen [1923] have used the method of oxidation by alkaline ferricyanide for the determination of amounts of glucose ranging from 0·02 to 0·36 mg. in 2 cc. Hanes [1929] has applied this method to the estimation of quantities of glucose and maltose up to 3·8 mg. in 5 cc.; also, Macleod and Robison [1929] have shown that the oxidation of reducing sugars by hypoiodite can be satisfactorily carried out in solutions of similar concentration.

The iodimetric method has already proved satisfactory for the determination of sugar in the apple if the oxidation is carried out on solutions containing about 0·06 g. sugar. It was therefore decided to investigate the oxidation of mixtures of glucose and fructose and of glucose and maltose by alkaline ferricyanide and alkaline iodine in concentrations of about 3 mg. per 5 cc.

Preparation of samples of pure sugar.

The glucose and fructose used were taken from the same samples as those employed by Archbold and Widdowson [1931] for an investigation of the iodimetric method.

Their rotatory powers and copper reducing values were determined:

Glucose.

$[\alpha]_D^{20°}$ (10 % solution) observed 52·73°; calculated 52·74°.

Weight of glucose taken	10·000 g. per 100 cc.
Weight of glucose (Cu value) found	9·998 g. per 100 cc.

Ash < 0·01 %.

Fructose.

$[\alpha]_D^{27°}$ (5·59 % solution) observed −88·19°; calculated (using Vosburgh's [1920] formula) −88·15°.

Weight of fructose taken	5·59 g. per 100 cc.
Weight of fructose (Cu value) found	5·52 g. per 100 cc.

Preparation of maltose. A sample of maltose, purchased as pure, was recrystallised twice by dissolving 25 g. in 200 cc. of 80 % alcohol heated in a water-bath under a reflux condenser. The solution was filtered, and sufficient alcohol was added to make 90 % concentration. The maltose was allowed to crystallise, filtered off and recrystallised in the same way. The maltose hydrate so obtained was dried *in vacuo* over sulphuric acid and then over phosphorus pentoxide until constant in weight, and finally dried at 50° until again constant in weight.

Copper reducing power (g. maltose hydrate per 100 cc. solution).

Calculated	Found
0·3771	0·3829

Optical rotation (calculated as anhydrous maltose).

$[\alpha]_D^{28°}$ (4·7146 % solution) Calculated 137·63 [Meissl, 1882]
Found 137·55

Oxidation of sugars by alkaline potassium ferricyanide.

The oxidation of the sugars by alkaline ferricyanide was carried out as described by Hanes, using sodium thiosulphate $N/75$ standardised against potassium iodate[1] solution and the following reagents:

[1] The potassium iodate was dried by heating at 100° for 1 hour. It was found that prolonged heating at 100° caused a slight discoloration of the salt. The thiosulphate was further standardised against potassium permanganate solution, and this gave a result differing only by 0·2 % from that given by the dried iodate.

A.	Potassium ferricyanide	8·25	g. per litre
	Anhydrous sodium carbonate ...	10·6	g. per litre
B.	Potassium iodide	12·5	g. in 500 cc.
	Zinc sulphate	25·0	g. in 500 cc.
	Sodium chloride	125·0	g. in 500 cc.
C.	Acetic acid	7·5 %	

·5 cc. sugar solution containing about 3 mg. sugar were measured into a glass specimen tube $1\frac{1}{4}'' \times 4''$ and 5 cc. of solution A were added. The tube was covered with a glass lid and placed in a boiling water-bath for exactly 15 minutes. It was then cooled in water for 1 minute and in ice for 2 minutes, after which 5 cc. of solution B and 2 cc. of solution C were quickly added, and the iodine liberated was titrated with $N/75$ sodium thiosulphate solution. Two drops of a 1 % solution of soluble starch in saturated sodium chloride were used as indicator. The tubes were left immersed in ice till the contents were titrated in order to prevent any loss of iodine. It was found convenient to carry out the estimations in batches of six. Blank determinations were made by substituting 5 cc. of distilled water for the sugar solution, and all determinations were carried out in triplicate.

Hanes used boiling-tubes $1'' \times 7''$ for his estimations, and apparently did not find it necessary to cool the tubes in ice to prevent loss of iodine. Using specimen tubes $1\frac{1}{4}'' \times 4''$ however, a series of blank determinations has shown that a loss of iodine equivalent to 0·06 cc. $N/75$ thiosulphate occurred if the tube containing the liberated iodine was allowed to stand for 10 minutes at room temperature, while the loss was equivalent to 0·33 cc. $N/75$ thiosulphate at the end of an hour. If the estimations are carried out in batches of six, 10 to 15 minutes will elapse between the titration of the liberated iodine in the first tube and the sixth, so that a serious error may be introduced by loss of iodine unless the procedure for cooling described above is adopted. The results of the determinations are shown in Table I.

Table I. *Loss of iodine on standing at room temperature.*

	$N/75$ thiosulphate required cc.
Titrated immediately	9·02
Titrated after 10 min. at room temperature	8·96
,, 30 ,,	8·79
,, 60 ,,	8·69
,, 10 in ice	9·02
,, 60 ,,	8·99

Sobotka and Reiner [1930] have investigated the oxidation of various sugars by alkaline ferricyanide, and obtained the same results as Hanes for glucose and maltose, confirming his observation that the reducing power of glucose increases slightly as the concentration of sugar increases, while that of maltose is practically constant. They also investigated the oxidation of fruc-

tose, and obtained a constant value for the reducing power, but for mixtures of fructose and glucose they found a lower reducing power than that obtained for either sugar alone. Callow [1930] found for invert sugar a simple relation between the thiosulphate equivalent and the invert sugar reduced, and his result differs only slightly from that obtained by Sobotka and Reiner for mixtures of glucose and fructose. Similar determinations were repeated under the conditions described above on a series of glucose, maltose and fructose solutions, containing weights of sugar varying from 0·5 to 3·5 mg. in 5 cc.

The results are shown in Table II.

Table II. *Oxidation of glucose, maltose and fructose by alkaline ferricyanide.*

	N/100 thiosulphate≡ ferricyanide reduced cc.	cc. N/100 thiosulphate/mg. sugar
mg. glucose		
0·671	1·99	2·97
1·342	4·04	3·01
2·013	6·09	3·03
2·684	8·23	3·07
3·353	10·36	3·09
mg. maltose (anhydrous)		
0·558	1·43	2·56
1·117	2·80	2·51
1·675	4·25	2·54
2·792	7·02	2·52
3·351	8·46	2·53
	Mean value	2·53
mg. fructose		
0·559	1·58	2·83
1·118	3·25	2·91
1·677	4·88	2·91
2·795	8·24	2·95
3·354	9·93	2·96

The results obtained show an increase in oxidation with concentration for glucose and fructose, and a constant value for maltose. For glucose and maltose the results are slightly higher than those found by Hanes, but repeated determinations, using every precaution to prevent loss of iodine always gave the same result. For fructose a definitely lower series of values is obtained than that observed by Sobotka and Reiner. In order to confirm this result determinations were carried out on invert sugar, obtained by dissolving 0·95 g. of sucrose in 150 cc. of water, boiling with 30 cc. of 0·5 N HCl for 1 minute, cooling, neutralising and diluting as required. The values obtained for the reducing power of the invert sugar estimated by oxidation with alkaline ferricyanide agreed very closely with those for mixtures of equal quantities of fructose and glucose and with the "mean value" for the sugars alone. The results are shown in Table III.

In describing the preparation of his sample of glucose, Hanes states that the sugar was dried *in vacuo* over concentrated H_2SO_4 for 48 hours. It has been found, however, that after 4 months *in vacuo* over P_2O_5, glucose still contains

2–3 % of water which can be driven off at 100°. Further, Hanes gives a value for the specific rotation of his sample of glucose $[\alpha]_D^{20°} = +52{\cdot}2°$ in 10 % solution.

Table III. *Oxidation of mixtures of equal quantities of glucose and fructose by alkaline ferricyanide.*

(N/100 thiosulphate≡1 mg. sugar.)

mg. sugar	Mean value for glucose and fructose estimated separately cc.	Mixture of glucose and fructose cc.	Invert sugar cc.
0·5	2·91	—	2·87
1·0	2·95	2·93	2·94
2·0	—	2·96	2·97
3·0	3·02	3·05	3·02

According to Tollens [1884] the theoretical value for a 10 % solution is 52·75°. (In the theoretical value 52·5° given by Hanes there is no correction for the concentration of the solution.) Both these observations suggest that his sample of glucose was not completely dry. Assuming that the glucose still contained 2 % of water his results would agree very closely with those shown in Table II.

Sobotka and Reiner give no details of the preparation of their samples of sugar, and it is difficult to account for the high reducing power obtained by them for fructose.

Oxidation of mixtures of glucose and maltose and of glucose and fructose by alkaline ferricyanide.

A series of determinations was next carried out on solutions containing mixtures of glucose and maltose and of glucose and fructose, the relative proportions of the sugars and the total concentration of the sugar in the solution being varied. For glucose and maltose the total sugar content of the solution was varied from 1 to 3 mg. in 5 cc., while the ratio of glucose to maltose was varied from 3:1 to 1:3. Then from pairs of results, the ferricyanide factors for glucose and maltose in the presence of one another were calculated by the method shown below.

	Wt. maltose (anhydrous) in 5 cc. mg.	Wt. glucose in 5 cc. mg.	Total wt. sugar in 5 cc. mg.	N/100 thiosulphate≡ ferricyanide used by 5 cc. cc.
1	1·117	1·071	2·188	5·95
2	0·558	1·607	2·165	6·21

$1{\cdot}117M + 1{\cdot}071G = 5{\cdot}95$
$0{\cdot}558M + 1{\cdot}607G = 6{\cdot}21$

$0{\cdot}653M + 0{\cdot}598G = 3{\cdot}32$
$0{\cdot}653M + 1{\cdot}795G = 6{\cdot}94$

$1{\cdot}197G = 3{\cdot}62$
$G = 3{\cdot}02$

$1{\cdot}607G = 4{\cdot}85$
$0{\cdot}558M = 1{\cdot}36$
$M = 2{\cdot}44$

G. Glucose factor = 3·02
M. Maltose factor = 2·44
} where M and G stand for the maltose and glucose factors respectively, *i.e.* cc. N/100 thiosulphate/mg. sugar.

The values for the series of estimations are shown in Table IV. The calculation shown above is made for experiment 5. It will be seen from the mean values obtained that the oxidation of one sugar is not affected by the presence of the other.

Table IV. *Oxidation of mixtures of glucose and maltose by alkaline ferricyanide.*

(*N*/100 thiosulphate≡1 mg. sugar.)

	Glucose cc.	Maltose cc.
1.	3·09	2·47
2.	2·93	2·52
3.	3·05	2·55
4.	2·96	2·59
5.	3·02	2·44
Mean	3·01	2·53

A similar set of determinations was carried out on mixtures of glucose and fructose, the ratio of glucose to fructose being varied from 1:1 to 1:5, whilst the total concentration of sugar in the solution used for estimation was maintained at about 3·0 mg. The factors for the oxidation of glucose and fructose in the presence of one another by alkaline ferricyanide were calculated from pairs of results as for the glucose and maltose, and a series of the results obtained is shown in Table V.

Table V. *Oxidation of mixtures of glucose and fructose by alkaline ferricyanide.*

(*N*/100 thiosulphate≡1 mg. sugar.)

Glucose cc.	Fructose cc.
3·11	2·99
3·13	2·97
3·15	2·97
Mean 3·13	Mean 2·97

The factors for both sugars are seen to be slightly higher than those obtained for either sugar alone, so that there appears to be a slightly increased oxidation of these sugars in the presence of one another if the ratio of fructose to glucose is greater than 1. It was found however that the use of the factors obtained for the sugars in their mixtures, when combined with the results obtained for the oxidation by hypoiodite, gave a value for the amount of fructose in a given solution which only differed by 1 % from the value for fructose when the factors obtained for the sugars alone were substituted in the calculation, while the calculated amount of glucose remained unaltered.

Oxidation of glucose, maltose and fructose by hypoiodite at 1°.

For the oxidation of glucose, maltose and fructose by hypoiodite the following reagents are required:

Sodium thiosulphate	$N/75$
Iodine in potassium iodide ...	$N/40$
Sodium hydroxide	0·3 %
Sulphuric acid	$N/4$

Samples of 5 cc. of sugar solution, containing about 3 mg. sugar are measured out into 2 oz. stoppered bottles which are left at 1° until the solutions have cooled to this temperature. 5 cc. $N/40$ iodine solution are then added followed by 2 cc. 0·3 % sodium hydroxide, and the bottles are placed in a water-bath maintained at 1° until the oxidation is complete. The stock solutions of iodine and sodium hydroxide are always kept at 1°, so that the temperature of the reaction mixture is not raised by their addition. When the reaction is complete the bottles are removed from the bath, 2 cc. $N/4$ sulphuric acid are added to each and the excess of iodine is titrated with $N/75$ sodium thiosulphate solution. All estimations are carried out in duplicate and blank determinations are made by substituting 5 cc. water for the sugar solution.

The oxidation of glucose to gluconic acid by hypoiodite at 1° has been found to be complete in 2 hours for a concentration of glucose about 0·06 % [Archbold and Widdowson, 1931]. The time of reaction for small quantities of glucose and maltose under the conditions described above was determined by allowing 5 cc. of solutions containing about 3 mg. of the sugars to react with hypoiodite at 1° for varying lengths of time. It was found that the oxidation of both sugars is complete in 2 hours and this period was accordingly used for all subsequent estimations.

The results are shown in Table VI.

Table VI. *Oxidation of glucose and maltose by hypoiodite. Time of reaction at 1°.*

Time of oxidation hours	$N/100$ thiosulphate ≡ I used cc.	cc. $N/100$ thiosulphate/mg. sugar
Glucose 3·353 mg. in 5 cc.		
$0\frac{1}{2}$	3·35	1·00
1	3·61	1·08
$1\frac{1}{2}$	3·71	1·11
2	3·72	1·11
	Theoretical value	1·11
Maltose 3·776 mg. (anhydrous) in 5 cc.		
$0\frac{1}{2}$	2·08	0·55
1	2·15	0·57
$1\frac{1}{2}$	2·21	0·58
2	2·23	0·59
	Theoretical value	0·59

Estimations were also carried out on solutions containing mixtures of glucose and maltose in varying proportions, and from pairs of results the iodine factors of the two sugars in the presence of one another were calculated by a method similar to that used for the calculation of the ferricyanide factors. A summary of the results is shown in Table VII. From the mean results it will be seen that the oxidation of one sugar by hypoiodite is not affected by the presence of the other.

Table VII. *Oxidation of mixtures of glucose and maltose by hypoiodite.*

(N/100 thiosulphate≡1 mg. sugar.)

Glucose cc.	Maltose cc.
1·11	0·59
1·09	0·60
1·11	0·62
1·13	0·56
1·13	0·56
Mean 1·11	Mean 0·59

The oxidation of small quantities of fructose alone by iodine and sodium hydroxide at 1° for 2 hours showed that the mean value of N/100 thiosulphate equivalent to 1 mg. sugar was 0·02 cc. For mixtures of glucose and fructose the oxidation of fructose was too small to be detected when less than 3 mg. of this sugar was present. For quantities of fructose above 4·0 mg. the fructose factor decreased with increasing quantities of fructose. The results are shown in Table VIII.

Table VIII. *Oxidation of fructose by hypoiodite at 1° for 2 hours.*

Fructose mg.	Glucose mg.	N/100 thiosulphate equivalent to fructose cc.	N/100 thiosulphate≡1 mg. fructose cc.
Oxidation of fructose alone.			
0·559		0·01	0·018
1·118		0·03	0·027
1·677		0·04	0·024
2·795		0·05	0·018
3·354		0·06	0·018
		Mean value	0·021

Fructose mg.	Glucose mg.	N/100 thiosulphate≡glucose (calculated) cc.	N/100 thiosulphate≡glucose + fructose (found) cc.	N/100 thiosulphate≡fructose cc.	N/100 thiosulphate≡1 mg. fructose cc.
Oxidation of fructose in the presence of glucose.					
1·677	1·607	1·78	1·78	—	—
2·236	1·071	1·19	1·19	—	—
2·795	0·536	0·59	0·60	—	—
0·559	0·536	0·59	0·60	—	—
1·118	1·071	1·19	1·18	—	—
0·839	0·161	0·18	0·17	—	—
1·677	0·321	0·36	0·35	—	—
4·974	1·281	1·42	1·56	0·14	0·028
7·461	1·921	2·13	2·29	0·16	0·021
14·922	3·842	4·26	4·46	0·20	0·013

The oxidation of fructose by hypoiodite can therefore be neglected in calculating the quantities of the sugars in solutions containing mixtures of glucose and fructose in which the amount of fructose is less than 3 mg.; the iodine value can therefore be taken as a measure of the glucose present.

Macleod and Robison [1929] applied the iodimetric method to small quantities of reducing sugars and obtained a considerable oxidation of fructose (9% in 20 min. at 21°) when sodium hydroxide was used as alkali. If sodium carbonate were used instead of the sodium hydroxide, however, fructose was oxidised only to a very small extent. The small oxidation of fructose (less than 2 %) here obtained when sodium hydroxide was used as alkali, as compared with the higher oxidation found by Macleod and Robison is probably due to the much lower temperature employed. Since satisfactory results could be obtained with sodium hydroxide at 1° its replacement by sodium carbonate was thought to be unnecessary.

The fructose-glucose ratio in the apple rarely exceeds 4:1, and in the young fruit for which this method was to be used the fructose and glucose are in approximately equal proportions, so a dilution of the cleared apple extract to bring it to a concentration of 3 mg. sugar per 5 cc. necessarily reduces the quantity of fructose present to a level at which its presence may be neglected.

The oxidation of solutions containing known weights of glucose and fructose was then carried out by both alkaline ferricyanide and hypoiodite, and by means of simultaneous equations the weights of glucose and fructose were calculated

$$1{\cdot}11x = \text{cc. } N/100 \text{ thiosulphate} \equiv \text{iodine reduced by 5 cc. sugar solution}$$
$$3{\cdot}13x + 2{\cdot}97y = \text{cc. } N/100 \text{ thiosulphate} \equiv \text{ferricyanide reduced by 5 cc. sugar solution}$$

where x and y are the weights of glucose and fructose in 5 cc. of solution.

The results given in Table IX show a maximum error of 2 % on the weight of fructose present, while the recovery of glucose is quantitative.

Table IX. *Oxidation of mixtures of glucose and fructose by alkaline ferricyanide and hypoiodite.*

	Glucose taken mg.	Glucose found mg.	Fructose taken mg.	Fructose found mg.
1	1·458	1·459	1·809	1·845
2	0·972	0·973	1·809	1·822
3	0·486	0·486	1·809	1·801
4	0·486	0·486	3·015	3·050

In sugar solutions containing sucrose estimations of the total sugar present in the solutions can be carried out by hydrolysing the sucrose with hydrochloric acid and re-estimating the reducing sugars by both methods of oxidation. Citric acid must not be used for the inversion since it is oxidised both by hypoiodite and by alkaline ferricyanide.

The combination of the two methods of oxidation, by alkaline ferricyanide and by hypoiodite, thus appears to give satisfactory results for the estimation of small quantities of glucose and fructose and glucose and maltose, and could probably be applied to mixtures of any two sugars provided that the difference

between the ratio of the factors of the two sugars obtained from each method of estimation is sufficiently large. The method is convenient since the same thiosulphate solution is used for both sets of titrations.

Hinton and Macara [1924] have shown that unreliable results are obtained for iodimetric determinations of quantities of glucose of about 0·06 g. unless there is added more than twice the quantity of iodine required to oxidise the sugar. Macleod and Robison [1929] state that they obtain satisfactory results for about 1 mg. sugar if 3–4 times the theoretical quantity of iodine is present.

Since the ferricyanide factor for glucose is about 3 times the iodine factor for quantities of sugar of about 3 mg., and for maltose the ferricyanide factor is 5 times the iodine factor, if aliquot samples of the same solution are used for oxidation by both methods under the conditions described, the quantity of iodine present is always more than three times the theoretical quantity required to oxidise the sugar present, so this condition is automatically fulfilled.

Investigation of the use of taka-diastase for the estimation of small quantities of starch.

The hydrolysis of starch in plant materials by taka-diastase instead of by mineral acid was investigated by Davis and Daish [1914], and it is now generally recognised that the older method of starch hydrolysis by boiling with dilute hydrochloric acid is quite valueless, because plant material contains a considerable quantity of pentose polymerides and other substances which yield reducing sugars when boiled with dilute acid. In addition, Davis and Daish pointed out that a destruction of glucose occurs on prolonged boiling with acid. This would introduce an error even if the preliminary hydrolysis were carried out with malt diastase since a subsequent acid hydrolysis is necessary to convert the dextrins which are produced to reducing sugars.

Davis and Daish showed that under suitable conditions, starch is converted quantitatively into glucose and maltose by taka-diastase, and that therefore no subsequent acid hydrolysis is necessary. The glucose and maltose present in the hydrolysis mixture were estimated by a copper reduction method and polarimetrically, and by means of simultaneous equations the quantities of glucose and maltose present were calculated, and hence the amount of starch. They found that the optimum conditions for the hydrolysis were 24 hours at 38°.

Horton [1921] repeated the work of Davis and Daish both on pure starch and on starch in the grain of wheat. His first result on pure potato starch was satisfactory, but he was apparently unable to repeat this, and the results he obtained in all later experiments gave a recovery of starch which was too low. He suggests that the conversion of starch to glucose and maltose is not quantitative but that a small quantity of dextrin still persists. He also finds a large variation in the glucose-maltose ratio, and concludes that different preparations of the enzyme vary in their maltase content.

Tottingham and Gerhardt [1924] obtained a smaller recovery of starch

with taka-diastase than with ptyalin from woody tissues and their difficulties appear to be similar to those encountered by Horton.

Thomas [1924] has stated that the ratio of glucose to maltose obtained by hydrolysis of starch with taka-diastase is constant, and therefore an estimation of the sugars in the hydrolysate by the picric acid reduction method gives a measure of the starch present.

Bish [1929] used taka-diastase in the determination of small quantities of starch in bracken rhizome, and he estimated the resulting mixture of sugars by Shaffer and Hartmann's [1921] copper reduction method. He claims that the results so obtained give comparative values for the starch content of the tissue. This method is only reliable if there is no variation in the glucose-maltose ratio.

In view of the differences of opinion with regard to the hydrolysis of starch by taka-diastase, the hydrolysis of pure starch by this enzyme has been investigated, and the application of the methods of oxidation by alkaline ferricyanide and by hypoiodite to the products of hydrolysis has been studied.

Purification of starch. A sample of maize starch was washed with cold water, alcohol and ether, and was dried at 100°. Duplicate weighed samples were then dried at 120°, one *in vacuo* and the other under atmospheric pressure, for 16 hours. It was found that more than 99·9 % of the water remaining after drying at 100° was removed at 120° at atmospheric pressure, and that after drying at 100° to constant weight the starch contained 3·575 % of water. Quantities of 0·3, 0·2 and 0·1 g. of purified starch, dried at 100°, were weighed out into 200 cc. conical flasks, and 100 cc. distilled water were added to each. The flasks were then heated in a boiling water-bath for half an hour, cooled to room temperature and 10 cc. of freshly prepared 1 % taka-diastase solution, 0·05 cc. of 5 % acetic acid and a little toluene were added to each. It had previously been found that 5 cc. of a 0·3 % solution of taka-diastase was sufficient for the complete hydrolysis of 0·3 g. of purified starch, but a preliminary trial showed that the minimum quantity of the enzyme required for the hydrolysis of a corresponding weight of starch in apple tissue was 0·1 g., so this quantity was adopted for the investigation of the hydrolysis of the purified starch.

The flasks were plugged with cotton wool and placed in an incubator at 38° for 24 hours. After removal from the incubator the solutions were heated to boiling to destroy the enzyme and to remove the toluene. They were then cooled and diluted to 500 cc. and the sugars in 5 cc. of the solution estimated as already described. A blank determination was carried out omitting the starch. The thiosulphate titration for the hydrolysed starch solution was corrected for the titration for the blank determination for the taka-diastase before calculation of results. Then by means of simultaneous equations the glucose and maltose present were calculated

$1{\cdot}11x + 0{\cdot}59y =$ cc. $N/100$ thiosulphate $\equiv$ iodine reduced by 5 cc. hydrolysate
$3{\cdot}01x + 2{\cdot}53y =$ cc. $N/100$ thiosulphate $\equiv$ ferricyanide reduced by 5 cc. hydrolysate

where x and y are mg. of glucose and maltose present in 5 cc. of the hydrolysis mixture. The amount of starch originally present was calculated by multiplying the estimated glucose by 0·9 and the maltose by 0·9479, and adding the products. An example of the calculation of results is shown below.

Calculation of results.

Wt. of starch dried at 100° = 0·1880 g.
Wt. of dry starch = 0·1813 g.
Diluted hydrolysate to 500 cc. Estimated sugar in 5 cc.

Thiosulphate (0·01303 N) ≡ ferricyanide reduced by 5 cc. of solution =	5·94 cc.
Thiosulphate (0·01303 N) ≡ ferricyanide reduced by 5 cc. of blank determination on taka-diastase =	1·51 cc.
Difference	4·43 cc.
Thiosulphate (0·01303 N) ≡ iodine reduced by 5 cc. of solution =	2·05 cc.
Thiosulphate (0·01303 N) ≡ 5 cc. of blank determination on taka-diastase =	0·52 cc.
Difference	1·53 cc.
N/100 thiosulphate ≡ ferricyanide reduced by hydrolysed starch in 5 cc. solution =	5·77 cc.
N/100 thiosulphate ≡ iodine reduced by hydrolysed starch in 5 cc. solution =	1·99 cc.

Then if x and y are the mg. glucose and maltose present in 5 cc. solution

$$1{\cdot}11x + 0{\cdot}59y = 1{\cdot}99$$
$$3{\cdot}01x + 2{\cdot}53y = 5{\cdot}77$$

$$3{\cdot}341x + 1{\cdot}776y = 5{\cdot}990$$
$$3{\cdot}341x + 2{\cdot}808y = 6{\cdot}405$$
$$1{\cdot}032y = 0{\cdot}415$$
$$y = 0{\cdot}402$$

$$0{\cdot}59y = 0{\cdot}24$$
$$1{\cdot}11x = 1{\cdot}75$$
$$x = 1{\cdot}577$$

$$\text{Starch originally present in 500 cc. solution} = \frac{(1{\cdot}577 \times 0{\cdot}9) + (0{\cdot}402 \times 0{\cdot}9479)}{10}\ \text{g.}$$
$$= 0{\cdot}1800\ \text{g.}$$

It was found that under these conditions more than 99 % of the starch could be accounted for; a series of results is shown in Table X.

Table X. *Hydrolysis of purified maize starch with taka-diastase.*

	Starch taken g.	Starch found g.	Starch found %	Glucose/ maltose
1	0·3184	0·3158	99·18	4·69
2	0·1813	0·1800	99·28	3·92
3	0·1431	0·1426	99·65	2·74
4	0·1386	0·1376	99·28	4·96

The glucose/maltose ratio obtained was higher than that found by Davis and Daish and was not constant even with two samples of the same preparation of enzyme. This bears out the observations made by Horton in this regard, though the variation obtained is not so great as that recorded by him. No indication of the persistence of dextrin in the hydrolysate as suggested by Horton was obtained, and it appears that the only products of hydrolysis are glucose and maltose.

The effect of clearing glucose-maltose and hydrolysed starch solutions with basic lead acetate and sodium phosphate.

Since this method for the estimation of starch was intended to be applied to apple tissue, a preliminary investigation was carried out on the effect of clearing the solution containing hydrolysed starch derived from this material. This work showed that satisfactory results could not be obtained unless the solutions were cleared before estimation of the sugars, so the effect of clearing on glucose-maltose solutions was investigated.

A solution containing about 0·3 g. glucose and 0·3 g. maltose was diluted to 1000 cc. and three aliquots of 200 cc. were further diluted to 250 cc. To one was added 0·5 cc. of basic lead acetate solution, followed by 1·5 cc. of saturated sodium phosphate, and to another was added 1·0 cc. of basic lead acetate and 3·0 cc. sodium phosphate before diluting to 250 cc. The third was diluted to 250 cc. without clearing. The solutions were filtered, and the sugar in 5 cc. of each was estimated by oxidation with alkaline ferricyanide. It was found that no loss of sugar occurred when clearing was carried out with these reagents. This agrees with results obtained with glucose-fructose solutions on the macro-scale [Archbold and Widdowson, 1931]. The results are shown in Table XI.

Table XI. *Effect of clearing glucose-maltose solutions with basic lead acetate and sodium phosphate.*

	$N/75$ thiosulphate ≡ ferricyanide reduced cc.
Uncleared	6·25
Cleared 0·5 cc. basic lead acetate	6·24
Cleared 1·0 cc. basic lead acetate	6·26

The effect of clearing on solutions obtained by hydrolysis of starch with taka-diastase was next investigated. 0·3 g. starch was hydrolysed as already described, and the solution diluted to 500 cc.; 200 cc. were cleared with basic lead acetate and sodium phosphate, filtered, and diluted to 250 cc. A second 200 cc. were diluted to 250 cc. without clearing. A similar pair of experiments was carried out on taka-diastase and 100 cc. of water, with no starch added. The reducing power of the sugar in 5 cc. of all four solutions was estimated by oxidation with alkaline ferricyanide and hypoiodite. The results are shown in Table XII.

Table XII. *Effect of clearing solutions of starch hydrolysed by taka-diastase.*

Results expressed as $N/75$ thiosulphate ≡ ferricyanide or iodine reduced by 5 cc. solution.

	Ferricyanide		Iodine	
	Cleared cc.	Uncleared cc.	Cleared cc.	Uncleared cc.
Taka-diastase blank	0·58	0·60	0·22	0·22
Starch	4·44	4·45	1·52	1·52

It was found that there was no precipitate when the lead acetate was added in either case although the solution became slightly turbid, and also, there was little or no difference between the values for the cleared and uncleared solutions. Hence it is unnecessary to clear solutions obtained by hydrolysing samples of pure starch before estimating the sugars. The presence of oxidisable material other than sugar in the aqueous extract from alcohol-insoluble apple residue after enzyme hydrolysis, however, necessitates clearing, but no loss of sugar occurs during the process.

It is thus evident that satisfactory results for starch can be obtained provided that no other polysaccharide present in the tissue under consideration is attacked by the enzyme. This point will be dealt with in a later paper.

Effect of boiling coloured apple extracts with "Suchar" on the ferricyanide and iodine oxidations.

It has previously been stated [Archbold and Widdowson, 1931] that the solutions from the evaporated alcoholic extracts from apples, after clearing with basic lead acetate and sodium phosphate, are colourless when first prepared, but gradually develop a brown colour on standing. They have also been shown to contain some material, other than sugar, which is oxidised by hypoiodite, but which has no effect on the copper reduction value of the solution. This oxidisable material can be removed by boiling the solution with charcoal before the iodine estimation is carried out. Solutions prepared from the alcoholic extracts of young apples are coloured immediately after clearing and become considerably darker on standing than those prepared from the more mature fruit. Accordingly, before proceeding to a series of estimations of the fructose and glucose in the extracts from the young apples, the effect on the ferricyanide and iodine oxidations of decolorising the brown solutions by boiling with charcoal was investigated. Coloured solutions were boiled for different numbers of times with "Suchar," a preparation of charcoal, and the sugars in the coloured and colourless solutions were estimated by both methods of oxidation. A difference of 0·66 cc. was obtained between the thiosulphate titrations for the ferricyanide oxidation of the coloured and the decolorised solutions, while the difference in the titrations for the iodine estimation was 0·24 cc. Decoloration is therefore necessary for both estimations. From the results obtained the apparent glucose and fructose present were calculated, and the results for one solution are shown in Table XIII.

Table XIII. *Effect of boiling with "Suchar" on a coloured apple extract.*

	% in apple	
	Glucose	Fructose
Unboiled	2·81	6·19
Boiled three times with "Suchar" (colourless)	2·37	6·22
„ five „ „	2·37	6·20

The effect of boiling with "Suchar" on solutions of fructose and glucose was next investigated to see if a measurable loss of sugar occurred during the process. Solutions of glucose and of glucose-fructose in the ratio 1:4, were left unboiled, boiled once, and boiled a number of times with "Suchar" and estimated by both ferricyanide and iodine methods.

The thiosulphate equivalent to the iodine reduced by 5 cc. of the solution decreased from 3·45 cc. for the unboiled solution to 3·35 cc. for the solution after boiling six times with "Suchar." The ferricyanide value also decreased from 9·71 cc. to 9·42 cc. This decrease in titration was equivalent to a 3 % loss of sugar. The results are shown in Table XIV.

Table XIV. *Effect of boiling with "Suchar."*

	mg. in 5 cc. solution	
	Glucose	Fructose
Glucose		
Unboiled	3·102	—
Boiled once	3·064	—
Boiled six times	3·009	—
Glucose + fructose		
Unboiled	0·838	3·071
Boiled once	0·838	3·030
Boiled four times	0·838	3·003

In the case of the glucose-fructose solution, in which the glucose only comprises about $\frac{1}{5}$ of the total weight of sugar present, there is no apparent loss of glucose after boiling the solution four times with "Suchar," but a loss of fructose of about 5 % occurs. Hence after boiling the solution four times with "Suchar" the ferricyanide value shows a decrease of 0·20 cc. while there is no change in the iodine value, since the oxidation of fructose by hypoiodite is negligible. In this case the effect of the adsorption of glucose by the "Suchar" is too small to be detected in the iodine titration, and the loss of reducing sugar appears to be entirely due to adsorption of fructose. This result is similar to that found for more concentrated sugar solutions, when the iodine value decreases 0·8 % after six boilings with "Suchar", while the reducing power estimated by copper reduction shows a loss of 5 % [Archbold and Widdowson, 1931].

The decrease in both the iodine and the ferricyanide values found after boiling the coloured apple extract with "Suchar" is much larger than can be accounted for by adsorption of sugar by the charcoal. From these results it was concluded that coloured solutions must be decolorised by boiling with charcoal before the sugars in them are estimated by alkaline ferricyanide or hypoiodite.

Determinations were carried out on a number of cleared solutions from evaporated alcoholic extracts of apples after boiling with "Suchar" till colourless, and diluting suitably for estimation. The values obtained for the percentage of glucose and fructose in the apple, determined by oxidation with alkaline ferricyanide and hypoiodite, as compared with the results for the same

samples estimated on a larger scale by copper reducing power and iodimetrically are given in Table XV.

Table XV. *Determination of glucose and fructose in apple extracts.*

	% sugar in apple tissue (wet weight).					
	Estimated by copper reduction and oxidation by hypoiodite (sugar solution 0·2 %)			Estimated by oxidation with alkaline ferricyanide and hypoiodite (3 mg. sugar per estimation)		
	Glucose	Fructose	Reducing sugar	Glucose	Fructose	Reducing sugar
1	1·28	2·70	3·98	1·23	2·77	4·00
2	1·19	3·32	4·51	1·17	3·32	4·49
3	1·28	3·77	5·05	1·22	3·74	4·96
4	1·26	3·98	5·24	1·21	4·08	5·29
5	1·46	4·25	5·71	1·39	4·30	5·69
6	1·58	4·36	5·94	1·51	4·38	5·89

Although with pure sugar solutions a loss of fructose and glucose occurs on boiling the solutions with charcoal, it will be observed that the results obtained for the total reducing sugar determined by copper reduction on the coloured unboiled apple extracts agree with those obtained on the decolorised solution by oxidation with ferricyanide, suggesting that the coloured substance is preferentially adsorbed by the charcoal. It does not therefore appear necessary to make a correction for any loss of sugar which occurs during decoloration. The percentage of glucose calculated from the small scale estimations is always slightly lower than that obtained from the more concentrated solutions. The differences are in no case more than 5 %.

Summary.

The Hanes modification of the Hagedorn and Jensen method for the determination of reducing sugars by oxidation with alkaline ferricyanide, when combined with the iodimetric method, has been satisfactorily applied to the estimation of small quantities of mixtures of glucose and fructose and of glucose and maltose.

These methods have also been applied to the determination of the glucose and maltose obtained by the hydrolysis of starch by taka-diastase. Tests with pure starch showed that the method was accurate to within 1 %.

It has been shown that there is no loss of sugar when glucose-maltose solutions are cleared with basic lead acetate and sodium phosphate.

It has been shown that cleared, coloured solutions obtained from alcoholic extracts of young apples should be boiled with a preparation of charcoal such as "Suchar" before estimating the fructose and glucose by oxidation with alkaline ferricyanide and hypoiodite.

The author wishes to take this opportunity of thanking Miss H. K. Archbold for her valuable suggestions and advice.

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XCIX. THE RESPIRATION OF *B. COLI COMMUNIS*.

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Cook, Haldane and Mapson [1931] investigated the oxygen-uptake of toluene-treated *B. coli* in presence of succinate, lactate, and formate. The toluene treatment restricts the oxidation of the first two substrates to a single stage, and thus renders the interpretation of the results easy. But it might be objected that the bacteria had been damaged by the toluene. For example the conclusion was drawn that the oxygenases concerned in the oxidation of the above substrates differed in their sensitivities to CO and HCN. This might conceivably have been due to the fact that, for reasons depending on cell structure, some were more accessible than others to toluene, and therefore more altered by it. We have therefore investigated the oxidation of several substances by the intact organism, besides making a few more observations in presence of toluene. The technique used was the same as that of Cook, Haldane and Mapson. Our N.T.C. strain of bacteria was used in $M/15$ phosphate buffer[1] at p_H 6·3 for formate oxidation, 7·6 for other substrates. All substrates were present in $M/60$ concentration, which is sufficient to give a maximum rate of oxidation in each case. Barcroft manometers were used throughout, and all experiments were carried out at 16°. When substrates were added, a bacterial suspension without substrate was placed in the left-hand cup. All experiments were done in duplicate.

Kinetics of oxygen-uptake.

In the absence of added substrate the rate of oxygen-uptake usually fell off during the first hour, and then became constant. (Fig. 1.)

In the presence of formate the curves obtained were very similar to those found in presence of toluene (Fig. 2). After 30 minutes the rate of oxygen-uptake became constant. If the experiments were continued long enough it fell off, but never increased. But in presence of glucose, succinate, and lactate, the curves obtained were concave upwards, *i.e.* the rate of oxygen-uptake increased with time, and ultimately became steady (Figs. 3, 4, 5). The reason for this is quite clear. The lactate, to take one example, is oxidised to pyruvate. This is not further oxidised in presence of toluene, so that the rate of oxygen

[1] The concentration of phosphate was incorrectly given in our former paper. 1·0 cc. of 0·2 M buffer solution was present in 3·0 cc. of mixture.

consumption does not rise. But the normal organism oxidises it. However, the amount of pyruvate produced in the first half hour or so at 16° is not nearly sufficient to saturate the catalysts concerned in its oxidation, so it, and prob-

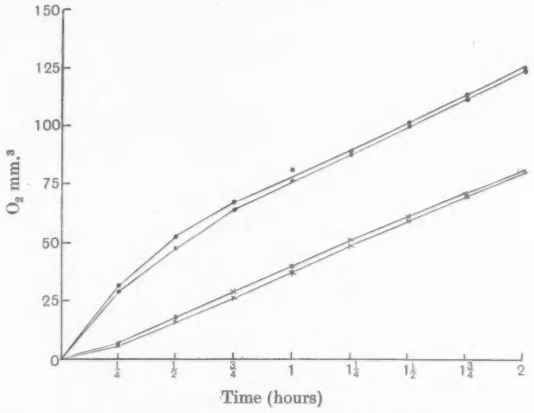

Fig. 1. O_2 consumption of washed *B. coli* (2·86 mg. N) in presence of air (upper curves) and a mixture containing 9 volumes of CO to 1 volume of O_2 (lower curves). p_H 7·6, 16°.

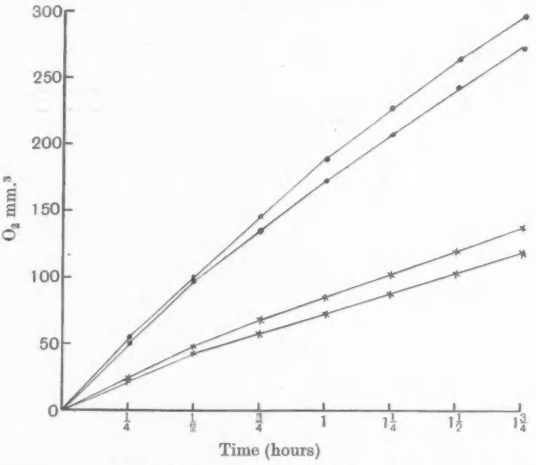

Fig. 2. O_2 consumption of *B. coli* (0·36 mg. N) + formate in presence of air (upper curves) and a mixture containing 3·88 volumes of CO to 1 volume of O_2 (lower curves). p_H 6·3, 16°.

ably further products of oxidation, continue to accumulate until, after an hour or so, a steady state is reached. At 40° the final steady state is reached in the first half hour or less, and no initial slow period is obvious.

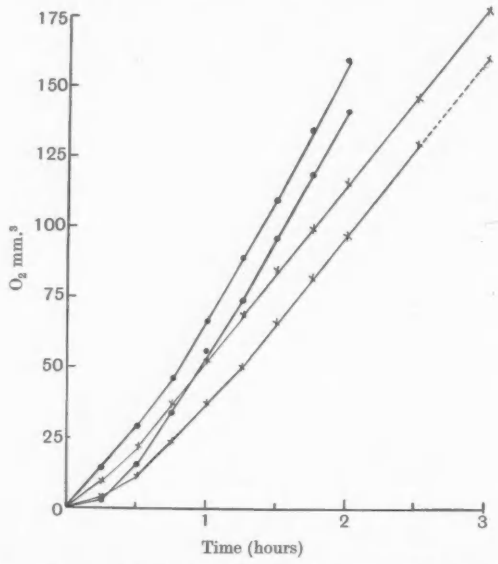

Fig. 3. O_2 consumption of *B. coli* (1·43 mg. N) + glucose in presence of air (upper curves) and a mixture containing 8·83 volumes of CO to 1 volume of O_2 (lower curves). p_H 7·6, 16°. Over the period represented by the dotted line the cup was strongly illuminated.

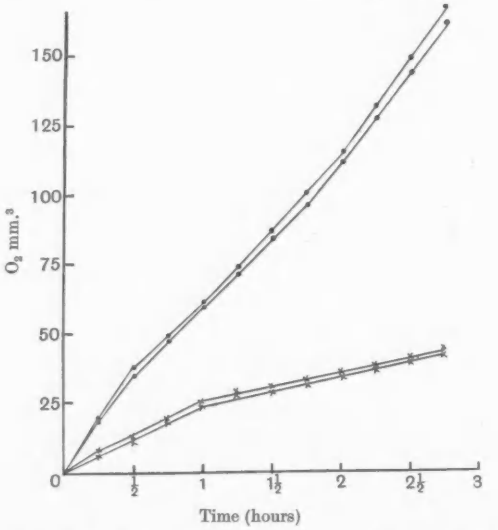

Fig. 4. O_2 consumption of *B. coli* (0·72 mg. N) + succinate in the presence of air (upper curves) and a mixture containing 9 volumes of CO to 1 volume of O_2 (lower curves). p_H 7·6, 16°.

The following calculation may make the matter clearer. Consider a substrate oxidised with constant velocity A, giving rise to a product in concentration x, and oxidised according to Henri's equation with velocity $\frac{Bx}{x+K}$. Then if v be the total velocity,

$$v = A + \frac{Bx}{x+K}, \text{ and } \frac{dx}{dt} = A - \frac{Bx}{x+K},$$

while $x = 0$ when $t = 0$. Eliminating x, and integrating,

$$\frac{(A-B)^2 t}{BK} = \frac{(A-B)(v-A)}{B(A+B-v)} + \log_e \frac{A(A+B-v)}{B(2A-v)}.$$

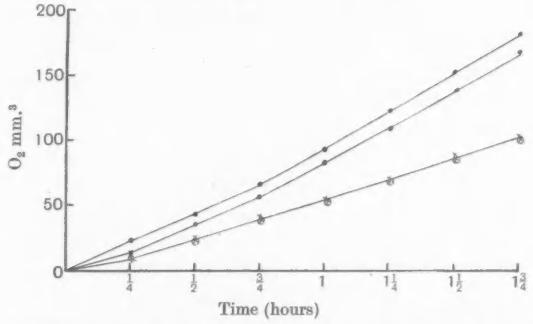

Fig. 5. O_2 consumption of *B. coli* (0·72 mg. N) + lactate in presence of air (upper curves) and a mixture containing 9 volumes of CO to 1 volume of O_2. p_H 7·6, 16°.

Hence if $A > B$, the final value of B is $A + B$, the concentration x of the intermediate increasing indefinitely. If $B > A$, the final value of v is $2A$, the final value of x being $\frac{AK}{B-A}$. In the cases here treated it appears that $A > B$. A similar calculation can be made if there are more than two steps in the oxidation.

We find that in the oxidation of glucose and lactate it is impossible to measure the initial velocity A with any great accuracy. Results are generally irregular in the first half hour, and the velocity is already increasing at the end of an hour. On the other hand with succinate no increase occurs until nearly two hours have elapsed. This is explained by the fact that fumarate, the immediate product of oxidation, must be hydrated to malate by fumarase before it can be further oxidised. There must be an accumulation, first of fumarate, then of malate. Only when enough of the latter has been formed will the rate of oxygen-uptake increase appreciably. In each case the final rate of oxidation exceeds the initial by 30–40 %.

Comparative rates of oxidation of different substrates.

Table I gives the final rates reached after two hours by the organism when washed free of substrate, and in presence of the four substrates employed. The results are expressed in mm.^{3} O_2 (reduced to 0° and 760 mm.) per mg.

Table I.

Substrate	None	Glucose	Succinate	Lactate	Formate
	16 (p_H 7·6)	72	95	158	472
	20 (p_H 6·3)	69	116	140	486
				131	
Average	18	71	106	143	479
Q_{O_2}	−217	−855	−1277	−1723	−5771

bacterial N per hour, and refer to a culture which had been kept for some weeks at 0° after washing. As these bacteria contain about 8·3 % of their dry weight of N, Warburg's Q_{O_2} is obtained on dividing by − 0·083. It will be seen that these values are very much larger than those found with yeast or mammalian tissues, where Q_{O_2} values of − 60 and − 20 respectively are normal. The contrast would have been still greater had our experiments, like most on yeast and tissues, been performed at 25° or 37°, instead of 16°. With fresh bacteria the rates of glucose and lactate oxidation are about the same. Thus in two experiments, one three days after the other, with a fresh suspension, we found O_2-uptakes per hour per mg. N of 162 and 124 mm.^{3} in presence of glucose, 170 and 126 in presence of lactate. It would seem probable that the enzymes concerned in the breakdown of glucose to more readily oxidised substances are less stable than the oxidising enzymes.

Some light is thrown on the nature of these products by experiments on fresh suspensions where glucose and lactate were oxidised alone and in mixture. The rates of O_2 consumption in mm.^{3} were:

Glucose	Lactate	Sum of rates	Glucose-lactate mixture
172·5	180·6	353·1	272·9
132·0	134·4	266·4	195·2

It will be seen that the oxygen consumption in presence of the mixture was 77 % and 73 % respectively of the sum of the rates in presence of glucose and lactate alone. On the other hand Quastel and Wooldridge [1927] found that the rate of methylene blue reduction by a glucose-lactate mixture was, if anything, slower than by lactate alone, which, as in our case, was a speedier reducer than glucose. The most obvious interpretation of our results is that some, but not all, of the glucose is transformed into lactic acid before oxidation. As the lactic dehydrogenase is already saturated with its substrate, the transformation of glucose into lactic acid does not increase the oxidation rate of the mixture. But about half the glucose appears to be converted into another substance (perhaps methylglyoxal) or substances, which are oxidised by different enzymes. This fraction cannot be calculated exactly, because substances may unite with enzymes which cannot activate them, thus inhibiting the oxidation of the proper substrate. It is conceivable that glucose may slow down the action of lactic dehydrogenase in this way. A comparison of our results with those of Quastel and Wooldridge suggests that the intermediary substances other than lactic acid reduce oxygen more readily than methylene blue in presence of *B. coli*.

for Q_{O_2} −217 −855 −1277 −1723 −5771
substitute Q_{O_2} −1·5 −5·9 −8·8 −11·9 −39·8

Line 3:
for dividing *substitute* multiplying

for the third and fourth sentences

Oxidations in presence of CO.

The oxygen-uptake of the starving organism during its first hour at 16° is considerably inhibited by the presence of 9 volumes of CO per volume of O_2 (Fig. 1). But the subsequent slow oxygen-uptake is hardly affected even when the ratio is raised to 15·2. Actually the duplicate uptakes in an hour without CO were 62·6 and 61·7 mm.^{3}, and with CO 57·5 and 57·6, giving a value of 190 for K, the apparent ratio of the affinities for CO and O_2. This agrees with Warburg's [1928] results with starved yeast, whose O_2 consumption is unaffected by CO.

Cook, Haldane and Mapson found that the toluene-treated bacteria gave very different values for K (the ratio of CO to O_2 giving 50 % inhibition) according to the substrate oxidised. Comparable results have been obtained with the untreated organism and are given in Table II.

Table II.

Substrate	K (toluene)	K (no toluene)
Glucose	—	19·9, 27·6
Succinate	6·3	5·8, 6·0
Lactate	9·7	9·5, 11·2, 13·7
Formate	2·3	2·4, 3·3

The method of calculation was the same as that given by Cook, Haldane and Mapson. With the exception of the value of 11·2 for lactate, all the above figures have a considerable probable error owing to inconstancy of oxidation rates, and occasionally to disagreement of duplicates. Little difference was found between the values during the initial phase of oxidation and the final, more rapid, phase. Thus the figure 19·9 for glucose is the mean of 19·0, obtained from the readings between 30 and 60 minutes from the beginning of the experiment, and 20·8, obtained from the readings between 90 and 210 minutes, over which period the rate of oxygen-uptake was nearly constant. It is clear that the values of K are, within the limits of experimental error, unaffected by toluene treatment. The oxidation of glucose is clearly less sensitive to CO than that of the other substrates, the small inhibitions obtained rendering an exact evaluation of K impossible. The slight inhibition is explained if the glucose is largely converted into lactic acid before oxidation. As the system is not saturated with lactate, the effect of CO is lessened, for, as Warburg points out, it is no longer the limiting factor.

Just as in the case of the toluene-treated organism, the inhibition by CO was practically unaffected by illumination with a fairly powerful lamp.

Oxidations in presence of phenylurethane.

When the 3 cc. of suspension included 0·5 cc. of water saturated with phenylurethane, we obtained, just as with the toluene-treated organism, only a slight inhibition. This amounted to 19 % in the case of glucose. When however CO was also present, its effect was greatly augmented. The value of K for

formate was lowered from 2·9 to 0·56. In the case of the toluene-treated organism it fell from 2·3 to 0·21. The effect without toluene was therefore comparable, though less marked.

Oxidations in presence of cyanide and other inhibitors.

The widely different sensitivity to cyanide of lactate and formate oxidation found in presence of toluene is equally characteristic of the normal bacteria. In 0·001 *M* KCN the rate of oxidation of formate was reduced to 4·6 % of the normal, that of lactate to 44 %. The corresponding figures in presence of toluene are 1·6 % and 39 %. In each case the figures for formate are a little uncertain, owing to the very slow rates, which could hardly be measured exactly. The great difference in susceptibility of the two oxygenases is evident.

In view of the fact that 2-aminophenol-4-sulphonic acid forms a complex with iron, and has been used by Krah [1930] to inhibit biological reactions believed to be catalysed by that element, it was thought possible that it might inhibit oxygenase activity. However 0·5 % of this reagent only reduced the rate of oxidation of formate by 12 %, which may not have been a specific effect. This does not of course disprove the presence of iron in oxygenase, but suggests that it is firmly bound there.

It was shown by Cook, Haldane, and Mapson that methylene blue could replace oxygenase as an intermediary between the dehydrogenases of the

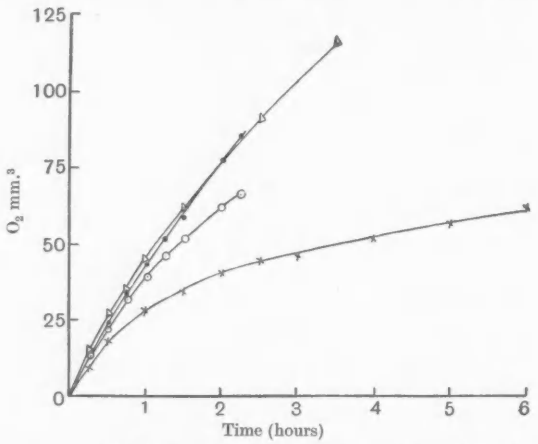

Fig. 6. O_2 consumption of *B. coli* (about 0·7 mg. N) + lactate, alone, and in mixtures containing cyanide, methylene blue and 8-hydroxyquinolinesulphonic acid (Q). p_H 7·6, 16°. Duplicate experiments agreed, but are omitted.

•—•—• Lactate control. △—△—△ Lactate + *M*/1000 KCN + *M*/750 M.b.
⊙—⊙—⊙ Lactate + 1/250 Q. ×—×—× Lactate + Q + M.b. + KCN.

toluene-treated organism and oxygen, when the oxygenase had been inhibited by KCN. The autoxidation of the methylene blue, which is catalysed by a

metal, can then be prevented by CO. We find that 8-hydroxyquinoline-sulphonic acid has a similar effect. Fig. 6 shows that this substance has little effect on the oxidation of lactate. But when, in presence of cyanide, oxygenase is replaced by methylene blue, the re-oxidation of leucomethylene blue, and hence the oxygen-uptake, is greatly slowed down by 8-hydroxyquinoline-sulphonic acid, the blue colour fading considerably. In the experiment here recorded the rate fell to 14 % of that in presence of methylene blue + cyanide. 8-Hydroxyquinolinesulphonic acid unites with copper, manganese, and nickel, but not iron. Hence our experiment confirms the view of Reid [1930] that the re-oxidation of leucomethylene blue is due to copper catalysis. It may also be regarded as a "model" illustrating the experiments of Hecht and Eichholtz [1929] in which tumour glycolysis, on the ground of its inhibition by various substances, was shown probably to include a catalysis by copper.

SUMMARY.

1. When *B. coli* oxidises glucose, succinate, and lactate (but not formate) at 16° the velocity of oxidation does not reach a maximum till more than an hour has elapsed, owing to the gradual production of further metabolites.

2. The rates of oxidation, especially of formate, are much greater than those caused by yeast or mammalian tissues.

3. The effects of CO and HCN on these oxidations differ greatly, as with the toluene-treated organism, thus confirming the view that at least three different oxygenases are present in the cell.

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Reid (1930). *Ber. deutsch. chem. Ges.* **63**, 1920.
Warburg (1928). Über die katalytischen Wirkungen der lebendigen Substanz (Berlin).

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for part 2 of summary
substitute The rates of oxidation are of the same order as those found for yeast and mammalian tissues.

C. A QUANTITATIVE METHOD FOR THE DETERMINATION OF VITAMIN C.

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From the Pharmacological Laboratories of the Pharmaceutical Society of Great Britain.

(*Received May 1st, 1931.*)

It has been shown by Zilva and Wells [1919], Höjer [1924, 1926], Goettsch [1928] and others that a scorbutic diet will produce histological changes in the teeth of young guinea-pigs. A transverse section of the root of the incisor of a young, normal guinea-pig shows a layer of long parallel odontoblasts surrounding the pulp and separated from the regular dentine by a narrow predentine. Fine canals cross the predentine and dentine from the odontoblasts. In a scorbutic tooth the odontoblast layer is disorganised, the predentine is calcified and an irregular layer of bone is formed between the predentine and the odontoblasts. The dentine canals are fewer and are only found radiating from the predentine outwards. Höjer described ten stages in the development of scurvy based upon the amount of disorganisation of the odontoblasts, the width and irregularity of the inner dentine or layer of bone, the degree of calcification of the predentine and the structure of the dentine. He expressed the amount of protection from the disease afforded by any dose, as a fraction of complete protection, and he claimed that this fraction represented the ratio of the test dose to the minimum dose which would give complete protection. Goettsch [1928] repeated Höjer's method and found that the great individual variations of the animals made it difficult to determine the fractions of the minimum protective dose. She could, however, find a dose such that all the animals receiving it were fully protected from scurvy and those receiving lower doses were partially protected in varying degrees. She compared the potency of two substances by determining the minimum dose of each which would produce complete protection in all the guinea-pigs given that dose. The ratio of the potency of the two substances was given by the inverse ratio of the minimum protective doses. Goettsch also determined the minimum protective dose of a substance by Zilva's method in which the diagnosis of scurvy is based on growth, haemorrhages of the joints, swelling and histological appearance of the rib junctions. She found that the minimum protective dose determined by Zilva's method was approximately one-half the dose necessary in Höjer's test. The ratio of the minimum protective doses of two substances was, however, the same in both tests. In these experiments no use could be made of results given

by animals receiving doses which were less than the minimum fully protective dose. If, as appears probable from the work of Goettsch, it may be assumed that using her diet the appearance of the teeth is entirely dependent upon the amount of vitamin C present, then it follows that the degree of scurvy produced must be graded to the dose of vitamin C, provided that sufficient animals are given each dose to eliminate differences due to individual variations. If a relation could be found between the average amount of protection given by a dose and the dose, then the dose which would produce full protection could be calculated for an unknown substance. The method used by us for determining the antiscorbutic potency of graded doses of orange juice was based on the results of Höjer and Goettsch.

The oranges were bought in London and stored at a temperature of about 5°. A fresh orange was cut each day and the juice was expressed and strained through muslin before being given to the animals through a graduated pipette. The guinea-pigs were imported one dozen at a time and were kept in the laboratory for 24 hours before the beginning of each test. During that period they were given a complete diet of oats, bran and plenty of greenstuff. The first batch of oranges was bought on November 7th, 1930, and used for feeding three dozen guinea-pigs which were received in the laboratory on November 6th, 1930, December 9th, 1930, and January 1st, 1931, respectively. The second batch of oranges was bought on January 23rd, 1931, and used for feeding two dozen guinea-pigs received on January 22nd and February 12th respectively.

All the guinea-pigs used for the test were given unlimited quantities of a basal diet consisting of bran 45 %, crushed oats 25 %, and dried skimmed milk 30 %, together with 1 cc. cod-liver oil twice weekly and tap-water *ad lib.*

Each set of one dozen guinea-pigs was divided into four groups of three animals and the members of each group received no dose, and daily doses of 0·75 cc., 1·5 cc., 3·0 cc. orange juice respectively. The weight of each animal was recorded twice weekly. The test was continued for 14 days, then all the animals were killed and examined for any macroscopic lesions. Their jaw-bones were placed in Jenkin's decalcifying solution. The growth response, *post mortem* observations and structure of the roots of the incisors are detailed below.

(*a*) *Growth responses.*

Guinea-pigs weighing between 250 g. and 350 g. were chosen for the test. The average initial weight was 293 g. In Fig. 1 composite growth curves are shown for the animals on each dose. Good growth was obtained in each group and there was no appreciable difference between the growth of the animals receiving only the basal diet and those receiving orange juice in addition. There is no evidence to show that the slower rate of growth of the control animals during the last few days of the test was in any way due to the onset of scurvy. Great variation was found in the growth of the individuals in each group, as will be seen from Table I.

Table I.

Daily dose of orange juice cc.	Greatest increase in weight of any individual in group g.	Least increase in weight of any individual in group g.	Average increase in weight g.
No dose	75	5	38
0·75	85	− 5	50
1·5	75	20	46
3·0	80	15	48

These results show that, for a test of such short duration as 14 days, the growth of a guinea-pig cannot be taken as any indication of the antiscorbutic value of its diet.

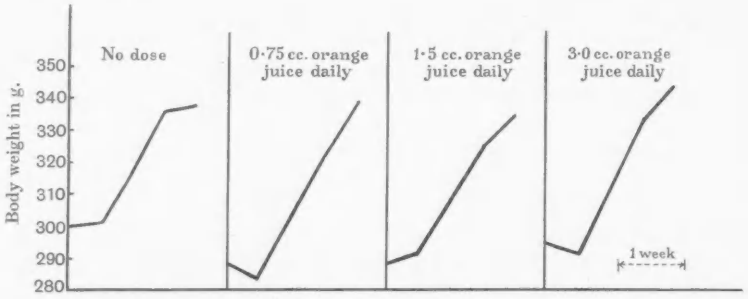


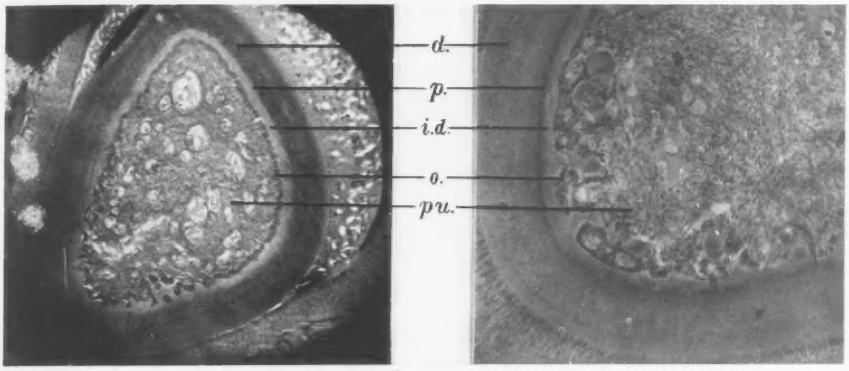
Fig. 1. Composite growth curves (average weights of 15 animals in each group).

(*b*) Post mortem *examination*.

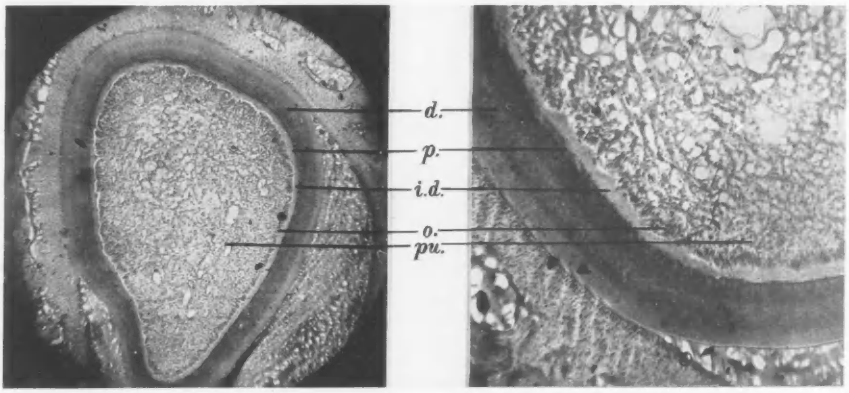
When they were killed all the 59 animals used in the experiment appeared to be in good, healthy condition except seven which had abscesses on the neck. Five others were found to have small neck abscesses when *post mortem* examinations were made. Special care was taken to note any haemorrhages at the knees or ribs and any swelling of the costochondral junctions of the ribs. These effects were, however, slight compared with those obtained in animals suffering from severe scurvy due to prolonged feeding on a deficient diet. The results are shown in Table II.

Table II.

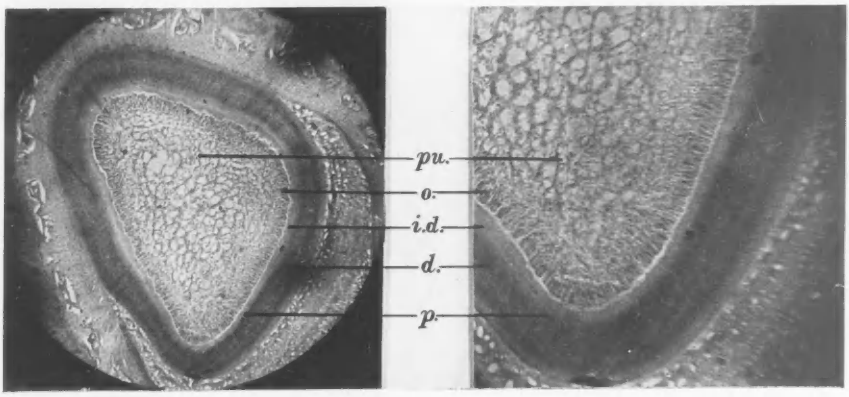
Daily dose of orange juice cc.	No. of animals showing any haemorrhages at joints	No. of animals showing swelling of rib junctions	No. of animals with abscesses on neck	Remarks
No dose	11	2	5	3 animals had slight congestion of lungs.
0·75	6	.	2	1 animal had slight congestion of lungs. 2 animals had slight infection in stomach and intestines.
1·5	3	1	3	1 animal had slight congestion of lungs.
3·0	5	.	2	2 animals had slight congestion of lungs.



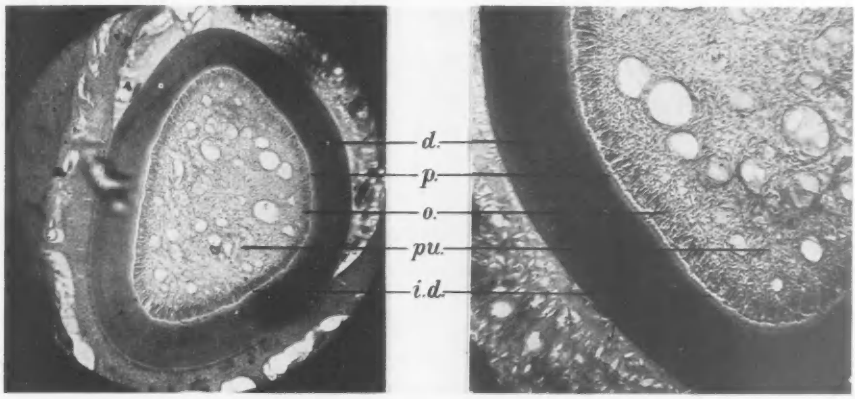
Degree of protection from scurvy 0



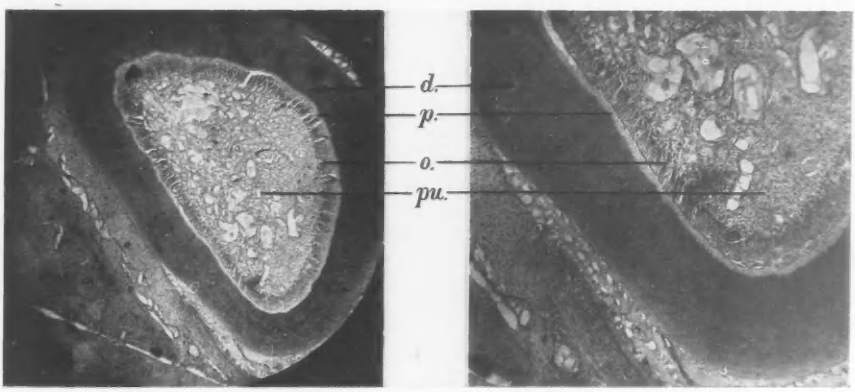
Degree of protection from scurvy 1



Degree of protection from scurvy 2



Degree of protection from scurvy 3



Degree of protection from scurvy 4

It will be seen that 11 of the 15 animals receiving no orange juice developed some haemorrhages at the joints. In the other groups the proportion of affected animals was lower. Similarly, two animals amongst those receiving no orange juice showed swelling of the rib junctions and five had abcesses on the neck. The corresponding figures for the other groups were lower. Thus macroscopic *post mortem* examinations showed that at the end of 14 days some difference could be detected between a scorbutic diet and a similar diet supplemented with orange juice, but no distinction could be made between the effect of different amounts of orange juice.

(c) *Structure of transverse sections of incisor roots.*

The method finally used for determining the relative potency of different doses of orange juice was based solely on the histological appearance of the roots of the incisors. In order to obtain a numerical value for the degree of scurvy produced in each animal, an arbitrary scale was devised in which the figures 0 to 4 represented stages from severe scurvy to complete protection from the disease. Plates IV and V show the characteristic appearance of transverse sections of the incisors in each group. A detailed description of the groups is given below and a summary will be found in Table III.

Group 0. In this group the odontoblasts were completely or almost completely disorganised. The inner dentine was wide, being approximately one-third the width of the dentine itself. It projected into the pulp and in some sections completely enclosed small portions of the pulp. The predentine was amorphously calcified and the Tomes's canals occurred only in the outer dentine. This picture represented the severest form of scurvy which we had produced in 14 days.

Group 1. The odontoblasts in this group were completely disorganised in some parts of the section, but in other places they were short and parallel, being only disorganised near the pulp. The inner dentine was narrower than in group 0 and although it was irregular near the odontoblasts, there were no long projections into the pulp. The predentine was calcified. In some of the animals included in this group the Tomes's canals occurred only in the outer dentine, in others a few canals could also be seen in the inner dentine.

Group 2. This group could be distinguished from the preceding ones in that the odontoblasts were all parallel though short and disorganised near the pulp. The inner dentine was narrow and regular, the predentine was either calcified or partially calcified and some Tomes's canals occurred in the inner dentine.

Group 3. The animals included in this group had only slight scurvy. The first part of the tooth to become affected by any lack of vitamin C in the diet appears to be the odontoblast layer. The cells, which are normally long and parallel, become disintegrated towards the pulp though still retaining their long and parallel formation. Teeth showing odontoblasts of this type were included in group 3 when there was no inner dentine and the predentine and Tomes's canals were normal. A few animals had teeth which were normal in

all respects except for a mere rim of inner dentine and slight disintegration of the odontoblasts. These were also included in this group.

Group 4. The animals in this group were completely protected from the onset of scurvy and consequently their teeth were normal. The odontoblasts were long and parallel. There was no inner dentine. The predentine was not calcified and the Tomes's canals crossed the dentine from the odontoblasts.

It was found that nearly all the teeth examined would fit into one of these five groups. There were a few exceptional animals in which one part of the tooth would indicate severe scurvy and justify inclusion in group 0, while another part of the section conformed with group 3 or 4. For example, a wide inner dentine was sometimes found accompanied by long, parallel odontoblasts, only slightly disorganised near the pulp. Such sections were judged separately by two individual workers and an average value for the degree of protection from scurvy was determined. In two teeth a series of two or three calcified rings could be seen in the dentine, resembling a series of calcified predentines. We could not understand the origin and interpretation of this structure.

In a normal tooth the dentine is often very wide, even approximately equal in thickness to the widest part of the pulp. In a scorbutic tooth the dentine is comparatively narrow, but since the width depends to some extent upon the exact position of the transverse section, this feature cannot be used as a criterion for determining the degree of scurvy.

Each guinea-pig used in the test was assigned a value for the degree of protection from scurvy in accordance with the scheme which is summarised in Table III. The results are shown in Table IV.

Table III.

Odontoblasts	Inner dentine	Predentine	Tomes's canals	Degree of protection from scurvy
Disorganised	Wide, with projections into pulp	Calcified	Only in outer dentine	0
Completely disorganised in places but some parallel formation	Narrow and irregular	Calcified	Only in outer dentine or mostly in outer dentine	1
All parallel but becoming disorganised near pulp	Narrow	Calcified or partially calcified	Mostly in outer dentine or crossing inner and outer dentine from odontoblasts	2
Long and parallel but becoming disorganised near pulp	Absent or mere rim	Not calcified	Cross dentine from odontoblasts	3
Long and parallel	Absent	Not calcified	Cross dentine from odontoblasts	4

The teeth of all the animals which received no orange juice showed severe or moderately severe scurvy and the degree of protection was estimated as

Table IV.

No dose		0·75 cc. orange juice		1·5 cc. orange juice		3·0 cc. orange juice	
Guinea-pig	Degree of protection from scurvy	Guinea-pig	Degree of protection from scurvy	Guinea-pig	Degree of protection from scurvy	Guinea-pig	Degree of protection from scurvy
1	0	4	0	7	3	10	Died on 4th day of test
2	0	5	2	8	1	11	4
3	0	6	1	9	0	12	3
13	0	16	2	19	3	22	4
14	0	17	2	20	3	23	4
15	0	18	2	21	3	24	4
25	1	28	0	31	2	34	3·5
26	1	29	1	32	3	35	3·5
27	1	30	1	33	2	36	4
37	1	40	1	43	3	46	4
38	1	41	2	44	4	47	4
39	1	42	1	45	2	48	4
49	1	52	2·5	55	3	58	4
50	1	53	3	56	1	59	4
51	1	54	1	57	3	60	4
Average value	0·6		1·4		2·4		3·9

0 or 1 according to the scheme in Table III. 3·0 cc. orange juice was the highest dose tested and this produced complete protection in 11 of the 14 animals used, and nearly complete protection in the remaining three. The effect of the lower doses of orange juice was more irregular. For example, among the 15 guinea-pigs given a daily dose of 1·5 cc. orange juice, one was assigned the value 0 and one the value 4, the remaining animals being given intermediate figures. It was assumed that the average value for the 15 animals receiving each dose represented the true protective power for that dose as measured by our arbitrary scheme. The average value for the degree of protection from scurvy was plotted against the dose of orange juice and a straight line was obtained as shown in Fig. 2.

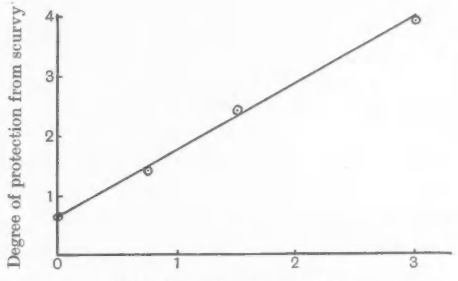


Fig. 2.

This curve could be used to compare any unknown substance with any standard. Suppose, for example, the potency of the unknown were required in

terms of decitrated lemon juice[1]. Some guinea-pigs would be given the unknown and others the lemon juice. The average degree of protection given by each dose of unknown and by each dose of lemon juice would be determined. Suppose the average degree of protection given by 1·5 cc. lemon juice were 2·4 and that given by a similar dose of unknown, 1·6, then the relation between the lemon juice and the unknown would be given by the ratio of the corresponding abscissae obtained from Fig. 2. Thus

$$\frac{\text{Potency of unknown}}{\text{Potency of lemon juice}} = \frac{0{\cdot}85}{1{\cdot}5} = 0{\cdot}6.$$

Orange juice compared with lemon juice as standard.

Orange juice is useful as a standard of reference for vitamin C since it is easily obtained and can be given directly to the animals without previous treatment. The guinea-pigs like the juice and drink it readily. Lemon juice is unpalatable unless decitrated and the process of decitration is difficult to control on the small scale. If insufficient calcium carbonate is added, a cloudy filtrate is obtained even after standing for two or three hours. If too much calcium carbonate is added, very little juice is obtained. Different lemons require different amounts of calcium carbonate for the best yield of decitrated juice. It is a disadvantage that a standard should require preliminary treatment of this sort, which differs for different samples of lemon juice. Orange juice, is, therefore, more convenient as a standard if it may be assumed that its potency is equally constant. In our experiments no difference in activity has been detected between the fresh oranges and those which had been stored for three months at 5°. Also there appeared to be no seasonal variation in the response of the guinea-pigs during the four months November to February. These results were, however, inconclusive and require further investigation. During the period from October 1928 to August 1930, 28 guinea-pigs were given a daily dose of 3 cc. orange juice. They were included in eight different experiments at different times of the year. All the animals were fully protected from scurvy except one which showed slight symptoms of the disease. It appears from these results that the antiscorbutic potency of orange juice is probably just as constant for different oranges as is the antiscorbutic potency of lemon juice.

Determination of the antiscorbutic potency of an unknown substance in terms of the potency of orange juice.

The dose of orange juice whose antiscorbutic activity is equal to that of a given dose of unknown may be determined from Fig. 2. The average value for the degree of protection from scurvy given by the unknown is found by experiment and the corresponding dose of orange juice is obtained directly from the curve.

[1] The use of lemon or orange juice as a standard of antiscorbutic potency has been provisionally recommended by a Sub-committee of the Committee on Accessory Food Factors appointed by the Medical Research Council and Lister Institute.

Exp. 1. In a test of Bramley's seedling apples in which three animals were used for each dose the following results were obtained:

Daily dose of apple = A g.	Degree of protection from scurvy shown by each animal on dose	Average degree of protection for each dose	Corresponding dose of orange juice = B (From Fig. 2) cc.	Dose of orange juice equivalent to 1 g. apple = B/A cc.
1·5	1 0 1	0·67	—	—
3·0	1 0·5 2	1·17	0·5	0·17
6·0	4 3 0	2·33	1·47	0·24

Thus the degrees of protection produced in the three animals receiving 3 g. apple were 1, 0·5 and 2 respectively. The average of these is 1·17. From the curve in Fig. 2 it will be seen that the abscissa corresponding with 1·17 degrees of protection gives 0·5 cc. as the equivalent dose of orange juice. The vitamin C content of 3 g. apple is, therefore, equal to that of 0·5 cc. orange juice and the dose of orange juice equal to 1 g. apple is 0·5/3 or 0·17 cc. Similarly 6 g. apple were found to be equivalent to 1·4 cc. orange juice, therefore, 1 g. apple is equivalent to 0·24 cc. orange juice.

Thus the antiscorbutic potency of 1 g. apple is approximately equal to that of 0·2 cc. orange juice. Greater accuracy could be obtained by the use of more animals on each dose. The error caused by individual variations must be very great when one animal out of the three receiving 6 g. apple gave the value 0 and another gave the value 4. In the following experiment five animals were used for each dose.

Exp. II. A sample of diluted tomato juice of unknown strength was examined for its vitamin C content. Daily doses of 3 cc., 6 cc., and 12 cc. were chosen and five guinea-pigs were used for each dose. The results were estimated independently by two experienced and two inexperienced observers. The results are shown in Table V.

Table V.

Dose of diluted tomato juice = A cc.	No. of guinea-pig	Values assigned by KK	GE	FD*	BM*	Average for each animal	Average for each dose	Corresponding dose of orange juice (from Fig. 2) = B cc.	Dose of orange juice equivalent to 1 cc. tomato juice = B/A cc.
3	61	4	4	4	4	4·0			
	62	2	1·5	2	1	1·6			
	63	0·5	0	1	1	0·6	1·4	0·7	0·23
	64	0	0	0·5	0·5	0·2			
	65	0·5	0	1	0	0·4			
6	66	3	3	3	2	2·7			
	67	0	0	0	0	0			
	68	4	4	4	3	3·7	2·2	1·4	0·23
	69	3	3	3	2	2·7			
	70	2	1·5	2	2	1·9			
12	71	4	4	4	4	4·0			
	72	4	4	3	4	3·7			
	73	3	3	3	3	3·0	3·7	2·7	0·225
	74	4	4	3·5	4	3·9			
	75	4	4	3·5	3·5	3·7			

* Observers with very little experience of normal and scorbutic teeth. Their estimates were given entirely from Plates IV and V and Table III.

It will be seen that the independent observations were in close agreement and that the three doses gave the same value for the potency of the tomato juice. This result is probably less accurate than the figures appear to indicate but the experiment shows that when five animals are given each dose, the differences due to individual variations are to a large extent eliminated.

Discussion.

The method described in this paper shows that graded responses can be obtained with different doses of vitamin C when the average protective value is determined for each dose. The curve relating the response to the dose may be used for determining the potency of an unknown substance in terms of any standard. This result is more accurate than that obtained from the minimum protective doses as recommended by Goettsch. Suppose, for example, an unknown fruit juice were examined for its vitamin C content. In order to determine the minimum protective dose, it would be necessary to test a wide range of doses, 0·5 cc., 1 cc., 2 cc., 4 cc. and 8 cc. might be suitable daily doses. Suppose three animals were used for each dose and all those receiving 4 cc. and 8 cc. were fully protected from scurvy while those receiving 2 cc. were not fully protected. Then the minimum protective dose of the unknown is less than 4 cc. but more than 2 cc. Since the minimum protective dose of orange juice is 3 cc., the ratio

$$\frac{\text{Potency of unknown}}{\text{Potency of orange juice}}$$ is greater than $\frac{3}{4}$ or 0·75 and less than $\frac{3}{2}$ or 1·5.

If the same fruit juice were examined by the method which is here described, fewer doses would be required though a wide range would still be necessary in order to obtain at least one dose which would give partial protection. Suppose the fifteen animals were divided into three groups of five individuals and the doses chosen for each group were 0·5 cc., 2 cc., and 8 cc. respectively. The following results might be obtained:

Daily dose of unknown = A cc.	Average degree of protection	Corresponding dose of orange juice = B (From Fig. 2) cc.	Dose of orange juice equivalent to 1 cc. of unknown = B/A cc.
0·5	1·3	0·6	1·2
2·0	2·9	2·0	1·0
8·0	4·0	Not < 3·0	Not < 0·4

Obviously 8 cc. is more than enough to produce complete protection. It is therefore more potent than 3 cc. orange juice. No further information can be gained from this dose. The lower doses show that the ratio

$$\frac{\text{Potency of unknown}}{\text{Potency of orange juice}}$$ equals approximately 1·1

since this is the average of the values in the right-hand column. This result agrees with that obtained by the minimum protective dose method but gives a more precise result.

Similar results could be obtained with decitrated lemon juice or any other standard.

This method is particularly useful for determining the antiscorbutic potency of substances containing little vitamin C. It is often difficult to give guinea-pigs high enough doses of such substances to produce complete protection from scurvy so that the minimum protective dose cannot be directly determined. Also when testing an unknown substance, a dose might be chosen which would produce partial but not complete protection. The corresponding dose of orange juice could be directly determined from the curve in Fig. 2.

SUMMARY.

A method is described for determining the antiscorbutic potency of a substance in terms of the potency of a standard such as lemon juice or orange juice. Different doses of orange juice were examined on a series of guinea-pigs and the amount of protection from scurvy produced in each animal was determined by means of an arbitrary scale. A curve was constructed relating the average amount of protection afforded to the dose of orange juice given. A straight line was obtained and this was used as a curve of reference for evaluating results on an unknown substance. Some disadvantages of the use of decitrated lemon juice as a standard are pointed out, and evidence is given which suggests that the antiscorbutic potency of orange juice is constant.

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DESCRIPTION OF PLATES IV AND V.

PLATE IV. *d.* dentine showing Tomes's canals. *p.* calcified predentine. *i.d.* inner dentine. *o.* odontoblasts. *pu.* pulp.

PLATE V. *d.* dentine showing Tomes's canals. *p.* uncalcified predentine showing Tomes's canals. *i.d.* inner dentine. *o.* odontoblasts. *pu.* pulp.

CI. THE ACTION OF DYESTUFFS ON ENZYMES.

II. FUMARASE.

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It has been shown in an earlier communication [Quastel and Wheatley, 1931] that there is a marked difference of behaviour between the various dyestuffs in their effects upon the oxidations of bacteria and tissues. Whereas certain dyestuffs such as ethyl violet or malachite green will eliminate almost entirely the ability of bacteria to oxidise such substrates as glucose, succinate, lactate or formate, others, *e.g.* Congo red, crystal scarlet, are inert. It is among the basic dyestuffs that the greatest toxicity is discernible; the acid dyestuffs appear to be comparatively inactive under the conditions employed. Not only, however, does the basic nature of the molecule play an important part in determining its toxic action; its molecular structure is of equal importance. It has been shown, too, that toxicity of a dyestuff towards oxidations is greatly influenced by the presence of substances which appear to have little to do with the oxidations themselves. Methylene blue, for instance, is much less toxic to the oxidation of succinate by bacteria or muscle in the presence of phosphates than in the presence of glycine or veronal at the same p_H, and it can be shown that bacteria 'poisoned' by methylene blue in the presence of veronal and unable to take up oxygen, will once again respire on the addition of phosphates. Phosphates appear to be able to combine with the dyestuff and to elute it from its combination with the oxidising system.

It is now proposed to describe the effects of dyestuffs on an enzyme, fumarase, which has a wide biological distribution. It is found in considerable amount in yeast, most forms of bacteria, red blood corpuscles, moulds, certain parts of plants and most animal tissues. The enzyme is entirely intracellular and it is owing to this fact and the circumstance that certain cells are not freely permeable to the fumarate molecule, that it is easy to make a wrong estimate of the total quantity of fumarase in the cell. Fumarase has been chosen for study not only because of the relative simplicity of the easily reversible hydrolytic reaction which it brings about but also because the experimental technique involved is simple and the effects of dyestuffs upon it can be determined with considerable accuracy.

Dyestuffs show a specificity of behaviour towards fumarase which is much

greater than that displayed towards the oxidations already referred to. Both basic and acid dyestuffs have toxic effects on the enzyme, a specificity of behaviour with both types of dyestuff being observable. The specificity, however, among the acid dyestuffs, is most marked—whereas, for instance, two such acid dyestuffs as crystal scarlet and orange G have no inhibiting action. Congo red is toxic at a molar concentration of $1{\cdot}2 \times 10^{-8}$.

Preparation of fumarase.

(*a*) *From* B. coli. It was shown in the first place by Quastel and Whetham [1924] that fumarase occurred in the cells of *B. coli*. Its action, however, was masked by the presence of reducing systems in the cell. When these systems were inactivated by growth inhibitors such as toluene or propyl alcohol the enzyme could be demonstrated in relatively large quantity [Woolf, 1929]. As will be shown later, however, it is necessary, in order to study the effects of certain substances on fumarase, to have a preparation of the enzyme free from the cell. This preparation can be made in the case of *B. coli* in the following way.

B. coli is grown in tryptic broth or on nutrient agar plates for 48 hours and the organism centrifuged from the broth or scraped from the plates with the addition of a little normal saline. It is washed twice with saline to remove extraneous material as far as possible and finally made up into a thick suspension with saline. To this suspension is added four times its volume of 1 % Na_2HPO_4, and this is allowed to stand in the incubator at 37° for three days. The suspension is now centrifuged and the centrifugate, which is slightly opalescent, mixed with a little kieselguhr and filtered. The clear filtrate contains the enzyme. It should be neutralised with a little acid and stored at 0°. (This preparation not only contains fumarase, but it has the property of transforming fumarate into *l*-aspartate in the presence of ammonium ions.)

(*b*) *From* M. lysodeikticus. If a washed suspension of this organism (prepared from a growth on agar plates) be mixed with fumarate in phosphate buffer and allowed to stand at 45° for six hours, little or no transformation into *l*-malate occurs. There would appear to be little or no fumarase present. If, however, as was shown by Penrose and Quastel [1930], the suspension of the organism be first lysed by the addition of saliva or a little egg-white, a rapid transformation of fumarate into *l*-malate takes place, the breakdown of the cell membrane bringing about a ready access of fumarate to the enzyme. Neither saliva nor egg-white enhances the activity of the cell-free enzyme. A method, therefore, of obtaining fumarase free from the cell is to lyse *M. lysodeikticus*, to centrifuge the solution after lysis and to filter the centrifugate after admixture with a little kieselguhr. The clear filtrate contains the enzyme, and more active preparations can be made in this way than by the method described for *B. coli*. The method, moreover, is far quicker, for the lysis of the organism takes place in a few minutes at 37° or 45°. The preparation is

practically free from phosphates and is convenient for the study of the actions of phosphates and salts on the enzyme.

(*c*) *From red blood corpuscles.* Clutterbuck [1928] reported that defibrinated ox blood showed a slight fumarase activity. This, I have found to be also the case with human, rabbit, or guinea-pig blood. Incubation of whole blood or a washed suspension of blood corpuscles with fumarate at 45° for several hours results only in a slight transformation into *l*-malate. If, however, the red blood cells be first lysed with distilled water then, just as in the case of the lysis of *M. lysodeikticus*, there is a most rapid transformation of fumarate into *l*-malate. Human blood proves, indeed, to be one of the richest sources of fumarase. (Further details of this, together with other facts concerning the biological distribution of fumarase, will be reserved for another communication.) The fumarase obtained from blood is accompanied by much protein, and this greatly affects results carried out with dyestuffs, *etc.*

(*d*) *From brain.* In estimating the effects of dyestuffs, *etc.* on fumarase it is of great importance to have a preparation of the enzyme which is reasonably free from protein and phosphates and which can be kept, as a standard preparation, for several months without losing its activity. None of the methods described above is particularly suitable. To obtain fumarase from bacteria involves the tedious procedure of preparing growths of the organisms, with subsequent lysis or autolysis, and the activities of the preparations are somewhat variable, depending upon the strain of the organism, effectiveness of lysis, *etc.*

Attempts were made to make a standard preparation from muscle but, although very active extracts were made, it was difficult to obtain a reasonably protein-free preparation which would keep for any length of time.

Finally the problem was solved by preparing the enzyme from brain. It is known that the enzyme occurs in brain, but perhaps it is not so well known that the white matter of brain is even richer in the enzyme than the grey. The whole brain, therefore, was used for the following preparation of fumarase.

The brain of an animal (I have used both sheep and human brain) is freed from the membranes and minced twice. To the minced brain is added twice its volume of 1 % Na_2HPO_4, and the suspension is well shaken mechanically for an hour. The resultant creamy liquid is filtered through muslin, and an equal volume of saturated ammonium sulphate solution is added and the suspension well stirred and allowed to stand overnight. A firm clot of protein, rich in fumarase, appears at the surface. This is poured on to muslin, which retains the clot. The clot in the muslin is allowed to drain for 24 hours and can be squeezed to facilitate the draining. Finally it is rapidly washed with water. (This clot may be suspended in water, which takes much of it into solution, and reprecipitated with saturated ammonium sulphate—the operation described above being repeated.)

The clot, which is of slimy consistency, is spread evenly over a glass plate and about twice its weight of plaster of Paris mixed with it. An even mixing

should be made so that the heat evolved, as the preparation dries, is quickly dissipated. Usually the preparation as a whole, if properly mixed and broken, does not become more than just warm—the temperature should not be allowed to become high enough to cause destruction of the enzyme.

The plaster of Paris preparation takes up water rapidly and should be stored in a refrigerator kept at 0° or slightly below 0°. I have found that this preparation will retain its fumarase activity for six months.

20 g. of the preparation is mixed with 100 cc. distilled water and well shaken in a machine for an hour. This is centrifuged and the slightly opalescent centrifugate is treated with a few cc. of $M/2$ potassium oxalate solution to precipitate all the calcium present. About 5 g. of kieselguhr are added to the suspension, which is shaken by hand for a few minutes and filtered. A perfectly clear filtrate with high fumarase activity is formed—this contains only slight traces of phosphate and protein. It is important, when eluting the enzyme from the plaster of Paris preparation, to use distilled water and not saline or phosphate or bicarbonate solutions. If the latter are used, preparations richer in fumarase are formed, but large quantities of protein are also taken into solution.

Fumarase preparations made in this way have remarkably constant fumarase activities, fresh extracts made every two or three days over a period of several weeks not varying in activity more than 5 or 6 %.

The plaster of Paris preparation may be extracted in a Soxhlet with ether without appreciably diminishing its fumarase activity—I have found, however, for my purposes, that the ether extraction is unnecessary.

This plaster of Paris preparation will be referred to in the remainder of this paper as the brain preparation.

Estimation of fumarase activity.

The estimation of *l*-malic acid may be easily accomplished by means of the polarimeter. Malic acid gives in acid solution and in the presence of molybdate a complex ion having very high rotatory power. Full details of the molybdate method of estimating *l*-malic acid are given by Auerbach and Kruger [1923]. These workers also describe the influence of various (not polarimetrically active) substances on the malic-molybdate complex. Citrates, for example, should not be present as they have the effect of greatly increasing the rotation. (This fact was not taken into account in the work of Mann and Woolf [1930] on the effects of citrates on fumarase activity and, in consequence, it is very difficult to interpret their results.)

I have made it a routine to take 5 cc. of the solution containing the *l*-malate, to add 1 cc. glacial acetic acid and 10 cc. of 14·2 % ammonium molybdate solution [see also Woolf, 1929], to filter and examine the solution polarimetrically in either a 1 dm. or a 2 dm. tube, using the mercury green line. When certain bacteria are being used, filtration after adding acetic acid and molybdate does not always give a clear solution—it is necessary then to centrifuge the solutions.

When using dyestuffs, the main difficulty is to remove the dyestuffs without affecting the content of *l*-malate. This, however, is easily accomplished, for all the dyes I have used, by shaking the solution, after addition of acetic acid and molybdate, with about 0·25 g. of decolorising charcoal. On filtering, a clear solution is obtained which is free from the dyestuff. Under these conditions the charcoal has an inappreciable effect on the *l*-malate content.

In the experiments to be recorded the enzyme is placed in presence of 0·08 *M* solution of sodium fumarate and incubation allowed to proceed at 45°. The rate of transformation of the fumarate into *l*-malate is linear for about 50 % of its transformation into the equilibrium concentration of *l*-malate. (Equilibrium is established at a ratio of about 1 fumarate to 3 *l*-malate.)

Using a 1 dm. tube, the rotation given, under the conditions specified, by a 0·08 *M* solution of fumarate which has reached equilibrium in the presence of fumarase at 45°, is 2·32°. The transformation of fumarate into *l*-malate is linear until the rotation begins to exceed 1·2°. Hence for studying the effects of various dyes, *etc.*, on fumarase, an amount of enzyme was taken, which would give in the time of experiment, and in the presence of 0·08 *M* fumarate, a rotation of about 1·2°.

2 cc. of the brain extract, prepared as described above, was found to give in the presence of 0·08 *M* fumarate and *M*/25 phosphate buffer, p_H 7·4, a rotation of 1·0 ° in 2 hours at 45°. The activity of the fumarase in the extract, after submitting it to the action of dyes, *etc.*, could be easily estimated, therefore, by determining the rotation under these experimental conditions. A rotation, for instance, of 0·5° would mean that an inhibition of 50 % of the activity of the enzyme had occurred.

Effects of dyestuffs on fumarase from brain.

Table I gives a representative series of results illustrating the action of dyestuffs at a fairly high concentration (1/2000) on the fumarase in brain

Table I. *Effects of dyestuffs (1/2000) on fumarase from brain in the presence of phosphate buffer,* p_H *7·4, at 45°.*

Dyestuff	% inhibition	Dyestuff	% inhibition
Congo red	100	Neutral red	0
Benzopurpurin	100	Janus green	81
Trypan blue	100	Safranine	4
Water blue	67	Methylene violet	4
Acid green	100	Bismarck brown	0
Crystal scarlet	5	Acriflavine	12
Orange G	0	Eosin	51
Methyl violet 6B	100	Erythrosin	100
Ethyl violet	100	Pyronine	0
Brilliant green	0	Methylene blue	0
Malachite green	38	Toluidine blue	9

extract. The experiments were carried out as follows. In each of a series of tubes were placed 2 cc. brain extract, 1 cc. *M*/5 phosphate buffer, p_H 7·4, and 1 cc. of an aqueous solution of dyestuff. The tubes were placed in a water-bath kept at 45° for 30 minutes, and then 1 cc. of a 0·4 *M* solution of sodium

fumarate was added to each tube. They were replaced in the bath for two hours, and then 1 cc. glacial acetic acid and 10 cc. of the ammonium molybdate solution were added to each tube. The malic acid was estimated polarimetrically in a 1 dm. tube after adding a little charcoal to each solution, shaking and filtering. Table I gives the percentage inhibitions of activity effected by each dyestuff on a particular extract. It is important, as will be explained presently, when comparing the inhibitions produced by various dyestuffs to use one extract for making the comparison.

It is evident from these results that both acid and basic dyestuffs are toxic to fumarase at a concentration of 1/2000 and p_H 7·4. There is, however, a marked specificity in action both among the acid and basic dyestuffs. Thus neutral red is inert but Janus green fairly toxic; orange G is inert but Congo red highly toxic.

It is interesting to compare these results with those obtained when studying the action of dyestuffs on oxidations [Quastel and Wheatley, 1931]. Here none of the acid dyestuffs was highly toxic. Among the basic dyestuffs, acriflavine, safranine and brilliant green were highly toxic; yet these dyestuffs have no effect on fumarase under the conditions employed.

Effects of dyestuffs at various concentrations on fumarase.

It is possible to obtain a better idea of the relative toxicities of the dyestuffs by studying their action at different concentrations. Table II shows the effects of a number of dyestuffs at considerable dilution, the experiments being carried out in the manner described above and upon one extract from brain.

Table II. *Percentage inhibitions effected by dyestuffs at varying concentrations on fumarase (in presence of phosphate buffer,* p_H *7·4, at 45°).*

	Concentration of dyestuff			
Dyestuff	1/16,000	1/32,000	1/64,000	1/128,000
Congo red	100	100	100	100
Benzopurpurin	100	100	100	100
Trypan blue	100	100	92	52
Methylene blue	12	0	0	0
Toluidine blue	10	6	0	0
Methyl violet	96	40	16	0
Ethyl violet	100	100	81	—
Crystal violet	82	21	0	0
Gentian violet	51	25	6	0
Brilliant green	7	0	0	0
Malachite green	20	10	0	0
Water blue	34	13	0	0
Neutral red	2	0	0	0
Janus green	48	8	0	0
Erythrosin	91	57	25	20

It is immediately clear that the dyestuffs most toxic at low concentrations belong to the Congo red series, *viz.* Congo red, benzopurpurin, trypan blue. Among the basic dyestuffs, those belonging to the triphenylmethane series are the most toxic, but these are not so active as the Congo red series. The acid dyestuffs of the triphenylmethane series, *e.g.* water blue, soluble blue, acid

green have a considerable toxicity but they are less active than the basic representatives. Erythrosin has a toxicity decidedly greater than that of eosin.

It is of interest to consider further the specificity of action of the dyestuffs. Let us compare Congo red (I), orange G (II), crystal scarlet (III) and Bismarck brown (IV).

$$C_{10}H_5(SO_3H)(NH_2)N:NC_{12}H_8N:N.C_{10}H_5(SO_3H)(NH_2)$$
I

$$C_{10}H_4(SO_3H)_2(OH)N:NC_6H_5$$
II

$$C_{10}H_4(SO_3H)_2(OH)N:NC_{10}H_7$$
III

$$C_6H_3(NH_2)_2.N:N.C_6H_4.N:NC_6H_3(NH_2)_2$$
IV

Whereas I is highly toxic, II, III and IV are inert under the conditions employed.

It is evident that the toxicity of I does not reside simply in its possession of sulphonic groups, or of amino-groups, or of the azo- or tetrazo-groups; nor is it likely that the size of the molecule is alone responsible. Possibly the benzidine nucleus is the effective group, but I have investigated the toxicity of benzidine itself and find it to be inert at a concentration of 1/1000. It would appear, therefore, that toxicity lies not in the possession of any simple grouping but on the arrangement of the molecule as a whole. The facts that benzopurpurin and trypan blue are also toxic indicate the great importance of the spatial arrangement of the groupings within the molecules—toxicity is conferred not by any single grouping in the molecule but by the relative arrangement of a number of groupings to each other.

It is evident that the toxicity of dyestuffs to the enzyme is of much the same type as that of dyestuffs and drugs to the living cell. The fact that enzymes can be so much more easily studied than the cell as a whole should be of considerable help in elucidating the factors determining toxicity of dyestuffs and drugs to the activities of the cell.

Effects of dyestuffs on fumarase extracted from bacteria.

The effects of dyestuffs upon fumarase prepared from *B. coli* and *M. lysodeikticus* in the manner described above are much the same as upon brain fumarase. The order of toxicities is similar. Methylene blue and toluidine blue, however, which have little toxic action on brain fumarase even at as high a concentration as 1/2000, will, at a concentration of 1/5000 entirely inhibit the fumarase prepared from *B. coli* or *M. lysodeikticus*. I have, so far, failed to discover the reason for this difference. On the whole, the fumarase from bacteria appears to be more sensitive to the action of dyestuffs than the preparation from brain. It seems probable that the differences between the extracts are due to differences in the nature of the substances accompanying the fumarase rather than to differences in the constitution of the fumarase prepared from differing biological sources. This, however, is a matter for further investigation.

Effects of dyestuffs on fumarase from blood.

The addition of dyestuffs appears to have no inhibiting action on the activity of the enzyme prepared from blood. Congo red, benzopurpurin, trypan blue, methyl violet, at concentrations as high as 1/5000 are without effect. It will be shown later that this inactivity of the dyes is due to the presence in the preparation of large quantities of protein.

Effects of the addition of mixtures of dyes to brain fumarase.

If a mixture of Congo red and methyl violet at the same concentrations be added to fumarase under the conditions described above, there is practically no toxic effect—though either of the dyestuffs alone is highly toxic at the concentration used. This result applies to many mixtures of dyestuffs. For instance, the mixtures of Congo red with crystal violet, methylene violet, methylene blue, or malachite green (where each constituent was at a concentration of 1/5000) gave percentage inhibitions of 14, 0, 5 and 6 respectively—the Congo red alone giving 100 % inhibition. On the other hand, mixtures of Congo red with water blue, crystal scarlet, or orange G were as toxic as Congo red itself.

Now it is observed that whenever a mixture of toxic dyestuffs is inert, a precipitate is formed—this being a compound or adsorption complex of the two dyestuffs. It seems that, in nearly all instances, the mixture of acid dyestuff and basic dyestuff forms a compound which is inert so far as its effect upon fumarase is concerned. On the other hand, a mixture of two acid dyestuffs or two basic dyestuffs is not less toxic than the more active of the two dyestuffs present.

The fact that acid and basic dyestuffs neutralise each other's toxic effects makes it evident that the acid or basic nature of the dyestuffs is a factor primarily involved in the combination with, or adsorption upon, the enzyme. It is clear, therefore, that the enzyme must possess acidic and basic groups (or groups bearing opposite charges), a result which is in keeping with the standpoint taken by Michaelis that an enzyme has ampholytic properties. It is significant, however, that both groups must be ionised, under the conditions of these experiments (p_H 7·4), and it follows that the active centre, responsible for the activation of fumarate, must have a zwitterion constitution.

Combination of enzyme and fumarate.

The important question now arises as to whether the fumarate molecule is itself adsorbed or combined at the basic and acidic groups which help to make up the fumarase centre. According to the active centre hypothesis, the adsorption (or combination) and the subsequent activation of fumarate at the centre will depend on the nature and spatial arrangement of the groups composing the centre. If, therefore, as the results with dyestuffs indicate, oppositely charged groups are constituents of the enzyme, and if such groups are con-

cerned with the adsorption and activation of the substrate molecule, the compound of fumarate with the enzyme should have the effect of preventing or inhibiting the combination of the dyestuff with the enzyme. Experiment entirely confirms this.

Exp. A. To 2 cc. brain extract were added 1 cc. 0·2 *M* phosphate buffer, p_H 7·4, 1 cc. water and 1 cc. 0·4 *M* sodium fumarate. After incubation at 45° for 2 hours the rotation was 1·23°.

To 2 cc. brain extract were added 1 cc. 0·2 *M* phosphate buffer, p_H 7·4, 1 cc. 1/3000 methyl violet solution and 1 cc. 0·4 *M* sodium fumarate. After incubation at 45° for 2 hours the rotation was 1·22°.

To 2 cc. brain extract were added 1 cc. 0·2 *M* phosphate buffer, p_H 7·4, and 1 cc. 1/3000 methyl violet solution. This mixture was allowed to incubate at 45° for 30 minutes, and then 1 cc. 0·4 *M* sodium fumarate was added. After incubation at 45° for 2 hours the rotation was 0·56°.

This experiment shows that incubation with methyl violet for 30 minutes prior to the addition of fumarate gave an inhibition of 54 %, whereas when the methyl violet was added together with the fumarate, no inhibition was effected.

Exp. B. The experiment was carried out as above but instead of methyl violet solution there was added 1 cc. 1/27,000 Congo red. The addition of fumarate together with the dye resulted in no inhibition of the enzyme; previous incubation for 30 minutes before the addition of the fumarate resulted in an inhibition of 53 %.

It is important, of course, in carrying out these experiments not to have too high concentrations of dyestuffs. The higher the concentration of dyestuff, the more difficult it is to observe the 'protective' action of the fumarate.

The experiments show that fumarate prevents the combination of both acid and basic dyestuffs at the enzyme. It would be expected, therefore, that the fumarate molecule is so arranged in its combination with the enzyme that it bridges both the acid and the basic groups. This arrangement (Fig. 1) of the fumarate molecule across two oppositely charged groups provides the mechanism required to polarise the double bond, this polarisation being necessary before activation can occur [Quastel, 1926]. Adsorption of the fumarate molecule is not in itself sufficient to bring about activation, for it is known that the fumarate molecule is absorbed at surfaces [Phelps, 1929], and there is no evidence that transformation to malic acid occurs. The fact, however, that the enzyme possesses two oppositely charged groups, to both of which the fumarate molecule is attached, must result in a polarising field which, in this case, is powerful enough to give the fumarate molecule the necessary energy of activation. Following polarisation, and the resulting induction of positive and negative charges at the ends of the double bond (or of an internal shift of protons and electrons), the addition of the elements of water occurs with the formation of malic acid.

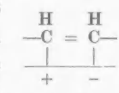

Fig. 1.

It is obvious that the type of molecule which can be adsorbed at the fumarase centre, so as to bridge the two oppositely charged groups must be very limited; for, considering the dyestuffs alone, where each dye presumably attracts only one of the groups, the specificity of adsorption or combination is most marked. The fact that such specificity occurs among the dyestuffs must mean that there are important factors bearing upon adsorption or combination at the enzyme, other than simply the presence of the positively or negatively charged groups.

The question which now arises is whether the double bond is essential for the adsorption of the fumarate (or its arrangement across the oppositely charged groups) at the enzyme. Preliminary work has begun on this matter and it is clear that succinate will also 'protect' fumarase from toxic dyestuffs. It is hoped to communicate details of the investigation later.

Effects of proteins on the toxic action of dyestuffs.

The addition of protein (egg-white, serum, gelatin) to fumarase results in a very marked protective action against toxic dyestuffs.

To 2 cc. brain extract were added 1 cc. 0·2 *M* phosphate buffer, p_H 7·4, 1 cc. 1/5 human serum, and 1 cc. 1/1000 Congo red solution. This was allowed to incubate at 45° for 30 minutes, and then 1 cc. 0·4 *M* sodium fumarate solution was added. After further incubation at 45° for 2 hours the rotation was 0·75°. In the absence of Congo red the rotation was 0·96°. Serum itself has no perceptible effect on the activity of fumarase under the conditions employed.

If the serum be heated, either at 100° for a few minutes or at 55° for 30 minutes, there is a marked increase in the 'protective' action.

Results with human and rabbit serum are shown in Table III. Egg-white, gelatin and milk have similar protective actions.

Table III. *Percentage inhibitions by dyestuffs on fumarase in the presence of sera and heated sera.*

Dyestuff	Serum	% inhibition
Congo red (1/5000)	None	100
,,	1/25 human serum	21
,,	1/25 human serum heated at 100° for 15 min.	0
,,	1/25 rabbit serum	65
,,	1/25 rabbit serum heated at 100° for 15 min.	10
,,	1/50 rabbit serum	80
,,	1/50 rabbit serum heated at 55° for 30 min.	29
Methyl violet (1/15,000)	None	47
,,	1/50 rabbit serum	0
,,	1/50 rabbit serum heated at 55° for 30 min.	0

The fact that the fumarase preparation from blood is not affected by dyestuffs is clearly due to the presence of the protein.

Now it is well known that proteins greatly diminish the activity of most antiseptics, and Hirschfelder and Wright [1930] have recently shown that the

attachment of dyestuffs, *e.g.* methyl violet, to egg-albumin follows the Freundlich adsorption isotherm very closely and have adduced evidence that the reaction between the dye and the protein is a surface (adsorption) reaction and not a strictly chemical one. By dialysis experiments they were able to estimate the amount of dyestuff taken up by protein. They then studied the effects of protein in reducing the antiseptic action of dyestuffs on yeast, and found that the reduction was not as great as would be anticipated if the compound between protein and dye exerted no toxic action. They therefore concluded that the fraction of the dye which was adsorbed on the protein exerted an antiseptic action.

In a number of ways the effects of proteins on the toxic action of dyestuffs on fumarase resemble those on the antiseptic action of dyestuffs on yeast. Hirschfelder and Wright found that denatured proteins adsorb more dyestuff than native ones, and, as shown in Table III, denatured sera have a greater protective action on fumarase. The most likely explanation of the protective action of proteins on the enzyme is that the protein adsorbs or combines with the dyestuff, leaving a relatively small quantity of dyestuff free to attack the enzyme. There is no evidence from my experiments that the combination of protein and dye has any toxic action on the enzyme. It seems strange that a combination of protein and dye should exert a toxic action on the yeast cell, as Hirschfelder and Wright maintain. These workers followed the action of the dye on the CO_2 production of the cell in presence of sucrose. Presumably the action of the dye occurs within the cell and if this is so, the cell membrane must be permeable to the compound of protein and dyestuff. There is, however, the possibility, it seems to me, that the presence of the protein has an effect on the permeability of the cell, making the entrance of the dyestuff easier and hence inducing a greater antiseptic action than would have been anticipated. A point in favour of this suggestion is that the yeast produces gas more freely in the presence of protein than in its absence, pointing, possibly, to an easier access of sucrose to the enzymes of the cell. This criticism is mentioned only to indicate the difficulty of interpreting results carried out on the intact cell.

Table IV. *Effect of varying quantities of human serum on the toxic action of Congo red (1/50,000) on brain fumarase, in the presence of phosphate buffer,* p_H *7·4, at 45°.*

Amount of serum present cc.	Rotation observed °
None (control with no dyestuff or serum)	0·91
0·1	0·91
0·05	0·90
0·025	0·77
0·0125	0·12
0·00625	0·00

Table IV shows the effect of varying amounts of serum on the toxic action of Congo red. It will be seen that in the presence of the larger quantities of the serum, Congo red has not the least toxic action—clearly, the compound of

Congo red and protein is inert. It is therefore possible to estimate, by the fumarase method, the actual quantity of dye taken up by the protein. The method simply consists in determining that quantity of protein which, in presence of the dyestuff, brings about, say, a 50 % diminution in the activity of the enzyme. The amount of free dyestuff which accomplishes this is known and hence knowing the original quantity of dyestuff present, a subtraction gives the amount of dyestuff combined with the protein. In this manner, it may be shown that 1 cc. human serum combines with approximately 3·5 mg. Congo red.

This method of estimating the combination of protein and dye (or drug) applies, of course, only to dyestuffs (or drugs) which are toxic to fumarase—it offers, however, definite advantages over the dialysis method.

Effect of a constant amount of dyestuff on varying quantities of enzyme.

Varying quantities of brain extract were placed in a series of tubes. To each of half of these tubes was added a quantity of Congo red to make up a final concentration of 1/100,000; the other half of the tubes received no dyestuff. 1 cc. phosphate buffer, p_H 7·4, and variable quantities of water were added to each tube to make up the final volume to 8 cc. The tubes were incubated for 30 minutes at 45°, and then 2 cc. of 0·4 *M* sodium fumarate solution were added to each tube. After a final incubation for 2 hours at 45°, the rotations were determined on 5 cc. Results are shown in Table V.

Table V. *Effect of a constant quantity of Congo red (1/100,000) on varying quantities of fumarase from brain.*

Quantity of enzyme cc.	Dyestuff	Rotation observed	Inhibition	Rotation / Quantity of enzyme
6	Absent	1·52°	—	0·25
6	Present	1·25	0·27°	0·21
5	Absent	1·30	—	0·26
5	Present	0·85	0·45	0·17
4	Absent	1·06	—	0·26
4	Present	0·40	0·66	0·10
3	Absent	0·80	—	0·27
3	Present	0·11	0·69	0·04
2	Absent	0·52	—	0·26
2	Present	0·00	0·52	0·00

It will be seen that, whereas in the absence of the dye the rotation is strictly proportional to the amount of enzyme, in the presence of the dye this proportionality entirely disappears.

It would have been anticipated that the greater the amount of enzyme, the greater the amount of combination between enzyme and dye and hence the greater the actual inhibition. The reverse of this occurs; the greater the amount of enzyme the less is the actual inhibition.

This result is easily explained if the assumption is made that the brain extract contains substances, other than the enzyme, which take up the dyestuff. The greater the amount of extract taken, the greater is the quantity of

impurity present which takes up dyestuff, and the smaller is the amount of the free dyestuff left over to attack the enzyme. Hence as the quantity of enzyme is decreased, the greater will be the apparent inhibition induced by the dye.

This can be placed on a mathematical basis taking the standpoint that the mass action law is followed or that the adsorption law is followed. Both laws give rise to curves which approximate closely to that found by experiment—but it is difficult from the data as yet available to decide which law is, in fact, followed.

The same phenomenon occurs with other dyestuffs and it seems to be a general rule, even with the purest extracts I have been able so far to prepare, that the actual inhibition effected by a dyestuff tends to increase as the amount of extract decreases.

It is for this reason that the comparison of the action of various dyestuffs should be carried out on one particular extract.

Comparison of effects of dyestuffs on the intact cell and on the cell extract.

When a washed *B. coli* suspension is used as a source of fumarase, the effects of the dyestuffs are, in certain instances, found to be quantitatively different from the effects on brain extract. It is shown in Table I that Congo red, benzopurpurin, methyl violet, ethyl violet and erythrosin at a concentration of 1/2000 will give 100 % inhibition of the activity of fumarase from brain. When a *B. coli* suspension is examined in precisely the same way as has been described for the extract, the following figures for the inhibitions are obtained (concentration of dyestuff, 1/2000).

Methyl violet	...	100 %	Congo red	...	56 %
Ethyl violet	...	100 %	Benzopurpurin	...	38 %
			Erythrosin	...	58 %

Thus, with a *B. coli* suspension the Congo red dyes are not as effective as the triphenylmethane series at these high concentrations.

When, however, an extract of fumarase is made from *B. coli* the dyestuffs show the same order of toxicity as with the brain extract.

A thick suspension of freshly grown and washed *B. coli* was incubated overnight at 37° with twice its volume of 1 % Na_2HPO_4. It was then centrifuged and the centrifugate (or extract) separated from the deposit; the latter was made into a suspension with normal saline. Both the extract and the suspension were brought to p_H 7·4. The suspension of the intact cells had about twice the furmarase activity of the extract. The effects of dyestuffs on the extract and on the suspension of intact cells were then compared—amounts of extract and suspension being taken so that they had equal fumarase activity. The results are shown in Table VI. It will be seen that, whereas methyl violet and water blue had practically the same effect on the intact cell

as on the extract, Congo red and toluidine blue were decidedly less toxic to the cells.

Table VI. *Comparison of percentage inhibitions effected by dyestuffs on fumarase in the cell extract and in the intact cell* (B. coli).

Dyestuff (1/5000)	Intact cell	Cell extract
Congo red	59	97
Methyl violet	88	82
Water blue	56	57
Toluidine blue	56	90

The most probable explanation of these results is that the bacterial cell has a much greater permeability to methyl violet and water blue than to Congo red or toluidine blue.

It would seem that the *B. coli* cell is almost freely permeable to members of the triphenylmethane series but resistant to those of the Congo red series, to toluidine blue and to erythrosin.

Reversibility of the compound of enzyme and dyestuff.

Very little success has so far attended efforts to remove the dyestuff from its combination with the enzyme.

Two methods have been tried.

(1) To the mixture of enzyme and toxic dyestuff which had been allowed to incubate at 45° for 30 minutes, there was added another dyestuff, sufficient in amount to combine with the toxic dyestuff present. The effect of the second dyestuff should be to increase the activity of the enzyme by removing the first dyestuff combined with it. Congo red and methyl violet were used in such concentrations that when added together no inhibition of the enzyme was effected. On adding the methyl violet, however, after the Congo red had been in contact for 30 minutes with the enzyme, the toxic effect of the latter was not appreciably decreased—the inhibition was 92 %.

(2) To the mixture of enzyme and Congo red, after incubation at 45° for 30 minutes there was added 1 cc. serum—an amount which would protect the enzyme from a considerable quantity of the dyestuff. A slight increase in the activity of the enzyme resulted. The inhibition without the serum was 100 %; the inhibition after the subsequent addition of serum was 79 %. Clearly, a slight reversibility was effected.

It is evident from these facts that the combination between enzyme and dyestuff is very strong and offers a striking contrast to that between the enzyme and its substrate (fumarate) where an easy reversibility must occur.

Effects of phosphates on fumarase.

In view of the action of phosphates on the toxicity of dyestuffs on the oxidations of bacteria and tissues [Quastel and Wheatley, 1931] it was of interest to investigate the influence of phosphates on the effects of dyestuffs

on fumarase. Now it has been shown by Clutterbuck [1928] and later by Mann and Woolf [1930] that phosphates increase the velocity of fumarate transformation by fumarase. On making a quantitative comparison of the action of phosphates on fumarase in *B. coli* and in brain extract (containing initially less than 0·02 mg. P per cc.), it was found that the effects were not identical. Whereas phosphates at a concentration of 0·04 *M* and p_H 7·4, approximately doubled the rate of fumarate transformation in presence of *B. coli* (an initial concentration of 0·08 *M* fumarate being used), phosphates under the same conditions increased the rate of change in presence of brain extract in a ratio of 1·3. Although there is no question that phosphates increase the rate of change of fumaric acid to *l*-malic acid in presence of brain extract, the effects are apparently not as great as with intact *B. coli*. Possibly this may be due to interfering substances in the brain extract, but the criticism is justified that in work on the intact cell, as carried out by Mann and Woolf, there are also complicating factors which should be considered in drawing conclusions as to the effects of phosphates or salts on the enzyme. It seems to me, for instance, that in the work on the effects of salts, *etc.* on p_H-activity curves using the intact cells, it is not altogether certain that the interior of the cell is at the same p_H as the external medium, especially when the latter is unbuffered.

Turning now to the question of the effect of phosphates on the action of dyestuffs, it is found that a protective action exists. The protection, however, does not seem to be as great as that observed in the case of oxidations. Methylene blue and toluidine blue (1/2000), which have little or no action on brain fumarase in the presence of phosphate (0·04 *M*), exert inhibitions of 15 % and 22 % respectively in its absence. Brilliant green, however, has a very toxic action in the absence of phosphates (100 % inhibition), this being removed by the presence of phosphates. Water blue and eosin also have smaller toxic effects in the presence of phosphate than in its absence.

Whether the phosphate acts by combining with the dyestuff or whether it acts by competing with the dye for a grouping in the enzyme is a question for futher investigation.

Summary.

1. Cell-free preparations of fumarase have been made from bacteria, red blood corpuscles and brain. A dry preparation has been made, by the use of plaster of Paris, which retains its fumarase activity at 0° for several months. The enzyme can be eluted from the powder by distilled water and is almost free from proteins and phosphates.

2. The effects of dyestuffs and mixtures of dyestuffs on fumarase are described. Both acid and basic dyestuffs are toxic. Among the acid dyestuffs, those of the Congo red series and of the triphenylmethane series are toxic, the former being the most effective. Congo red is toxic at a concentration of $1·2 \times 10^{-8}$ *M*.

3. The differences in action of dyestuffs on bacterial fumarase and brain fumarase are described.

4. Fumarate 'protects' the enzyme from toxic dyestuffs, *e.g.* Congo red or methyl violet.

5. The bearing of these facts on the constitution of the enzyme and its mode of action are discussed.

6. Proteins 'protect' fumarase from dyestuffs by combination with them, the compound of protein and dye being inert. By this means it is possible to estimate the amount of dye taken up by the protein. Denatured protein takes up more dyestuff than native protein.

7. The actual inhibition decreases as the amount of enzyme increases, when a constant amount of dyestuff is present. This is explained on the assumption that the extracts contain substances, other than fumarase, which combine with the dyestuff.

8. Differences between the intact cell (*B. coli*) and the extract from the cell are described. Permeability of the cell membrane plays an important part in determining toxicity of a dye to the enzymes of the intact cell. Whereas *B. coli* is almost freely permeable to members of the triphenylmethane series, it is resistant to Congo red, toluidine blue and erythrosin.

9. The combination of enzyme and dyestuff is not easily reversible.

10. Phosphates exert some protective action on the enzyme against toxic dyestuffs.

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CII. THE EFFECT OF VITAMIN B DEFICIENCY UPON THE VITAMIN A RESERVES OF THE RAT.

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In a previous communication [Moore, 1931] it was shown that when rats are given diets rich in carotene, vitamin A may accumulate in the liver in amounts that seem fantastically great when compared with the minimal daily requirements of the animal. Further, if a rat which had been fed on carotene in this way were then placed upon a diet free from vitamin A the reserves of that factor did not materially decrease after a lapse of 60 days. These findings confirmed the current view that the liver possesses the power of concentrating vitamin A and storing it over prolonged periods without deterioration. In distinct contrast the fats of the other organs, together with the general storage fat of the body, continued to contain only small traces of the vitamin.

This one-sided distribution of the vitamin A reserves suggested the problem which forms the subject of the present communication. It is well known that a rat subsisting upon a diet which is deficient in the vitamin B complex loses weight progressively, and that this loss of weight is accompanied by disappearance of the body fat. In so far as the writers are aware, however, no observation has been placed on record to show whether this loss of fat is accompanied by a corresponding loss of fat-soluble vitamins. It was considered of interest, therefore, to examine the vitamin A reserves of the liver under these conditions, especially since two distinct possibilities seemed open. On the one hand, it might be supposed that in the severe emaciation resulting through vitamin B deficiency the great superfluity of vitamin A, serving no obvious purpose, would be dispersed. On the other hand, the vitamin A reserves of the liver, being concentrated there by means of some mechanism quite distinct from that regulating the decomposition and resorption of storage fat, might be expected to show no change, at least so long as the liver itself remained undamaged. This second hypothesis received *a priori* support from the observation of Drummond and Hilditch [1930] that the vitamin content of cod-liver[2] is not influenced by the variations in fat content associated with the sexual cycle, and in the present paper it is shown to be correct.

[1] In the whole time employment of the Medical Research Council.

[2] It may be pointed out that the liver of the cod may contain almost 40 % of fat, whereas in mammalian livers the fat content does not exceed about 2 %. The liver in the cod must therefore play a much more important part in the economy of stored fat than is the case with mammalian liver.

Experimental.

The experimental animals used were adult albino rats which had been reared to maturity upon a complete synthetic diet and subsequently maintained for a prolonged period (about 200 days) upon a diet rich in carotene ("light white casein" 20 %, rice starch 60 %, red palm oil 15 %, salt mixture 8 %, dried yeast 15 %, (additional) radiostol 1 drop per rat daily). Under these conditions the vitamin A reserves of the liver, as previously reported [Moore, 1931][1], had been found to be remarkably constant between individuals, the usual value being about 70,000 blue units. The experimental procedure consisted of the exposure of the rats to diets deficient in the vitamin B complex until severe emaciation had resulted. The animals were then examined *post mortem* both in regard to the extent of the remaining body fat and the amount of vitamin A still contained in the liver oils.

Vitamin A reserves of the rat when fed upon a complete diet with excess of carotene. Rat 1 ♀. This animal, weight 220 g., served as a control. It was killed during the present experiment, without changing from the complete red palm oil diet, in order to ensure that the vitamin A reserves of the batch of rats used should not depart materially from the value expected through previous experience. The liver only was examined.

	Weight of fat mg.	Total natural yellow units	Total $SbCl_3$ blue units
Liver	154	150	70,000

Persistence of the vitamin A reserves during continued feeding of carotene accompanied by vitamin B deficiency. Rat 2 ♂. From the complete diet containing red palm oil this rat was transferred to a diet deficient in the vitamin B complex which still contained red palm oil (Glaxo caseinogen 20 %, cane sugar 60 %, salts 5 %, red palm oil 15 %). On this diet it declined rapidly and died after 48 days, having decreased in weight from 378 g. to 171 g. *Post mortem* examination showed complete absence of fat depôts and general emaciation. Only the liver was analysed for fat and vitamin A.

	Weight g.	Weight of fat mg.	Total natural yellow units	Total $SbCl_3$ blue units
Liver	6·7	114	130	110,000

Persistence of vitamin A reserves on a diet lacking both vitamin A and vitamin B. Rat 3 ♀. From the complete diet containing red palm oil this rat was transferred to a diet similar to that given to rat 2 with the exception that arachis oil now replaced the red palm oil as the fat component. The diet was therefore completely free from vitamins. The body weight fell from 266 g.

[1] The method employed in the extraction of tissues, although essentially the same as that previously described [Moore, 1930], has now been modified to permit more rapid estimations. By the addition of 50 % of alcohol to the alkaline solution of the tissue it has been found possible to remove nearly all the fat by one extraction with about 3 volumes of ether. After washing, the ethereal extract is dried by filtering through a layer of anhydrous sodium sulphate contained in a funnel provided with a sintered glass diaphragm.

to 168 g. in 35 days when the animal, being extremely emaciated, was killed. *Post mortem* examination revealed that the remains of the fat depôts were very scanty. The liver was dissected out and examined in the usual way. In addition the remainder of the carcase, including the pelt and intestinal tract and contents, was extracted with a view to finding the actual amount of fat present.

	Weight g.	Weight of fat	Total $SbCl_3$ blue units
Liver	6·8	120 mg.	68,000
Carcase	161	9·0 g.	Very low

Rat 4 ♀. This animal received the same diet as the preceding one but was killed after the slightly longer period of 41 days, the body weight falling from 202 g. to 126 g. At the end of this time it appeared extremely emaciated but was still active. The autopsy revealed a complete absence of intraperitoneal fat. Analyses were made separately of the liver, the intestinal tract and contents, the pelt and the remainder of the carcase.

	Weight g.	Weight of fat mg.	$SbCl_3$ blue units
Liver	5·2	78	70,000
Intestinal tract	13·2	181	200*
Pelt	19·6	398	0
Carcase	85·1	114	0

* The meaning of this positive result is not clear, but faulty dissection may be suspected. This value could be accounted for by the unintentional inclusion of about 20 mg. of liver tissue.

Spectroscopic data. In the case of the liver oils examined above, the blue colour produced in the $SbCl_3$ reaction was characterised by the prominent band at 610–630 $\mu\mu$ which is associated with vitamin A. The natural yellow colour, when not stated, was not more than a trace. The chromogen present must therefore have consisted almost exclusively of vitamin A.

Discussion.

It is at once evident from the data given that the remarkably high reserves of vitamin A which the rat is able to store up when fed on a diet rich in carotene are not depleted when it is concurrently or subsequently suffering from a lack of the vitamin B complex. The figures for rats 2, 3 and 4 show that in the state of extreme emaciation following on a deficiency of the vitamin B complex the liver oils still contained the characteristically very high vitamin A content observed by Moore [1931]. At the same time, the observation that the liver oil of rat 2 was of a normal pale colour indicates that vitamin B deficiency in no wise diminishes the power of the animal to transform carotene to vitamin A when the pigment is present in the diet in very great excess of the animal's requirements.

The data for rat 4 are particularly instructive in showing how far the loss of body fat can go without materially affecting the large vitamin A store of the liver. The total amount of fat which could be extracted from the four parts into which the body was divided was less than 1 g. The liver fat may

have been depleted (it is not quite certain whether the low figure of 78 mg. is sufficiently below the normal one of approximately 150 mg. to allow of this conclusion being drawn) but there is no doubt that the vitamin A store of the liver remained unaffected, since the figure of 70,000 blue units obtained is representative of those found for rats still receiving the complete red palm oil diet.

These results confirm the view that the vitamin A reserves of the liver constitute a system quite apart from the general fat reserves of the body. To complete the picture mention may be made of a complementary condition which is sometimes encountered in rats suffering from avitaminosis A. It is certainly true that rats surviving during long periods of decline in this condition become no less emaciated than those suffering from avitaminosis B, but when death is more sudden quite large fat deposits may remain even though no traces of vitamin A can be detected colorimetrically either in the liver or in the body fat. Thus the body of a young male rat, typical of several others, which died rather suddenly at a weight of 174 g. after 61 days' exposure to a diet deficient in vitamin A, showed almost normal fat deposits, amounting to 16·2 g., while the liver yielded 144 mg. of oil. Nevertheless, both the body fat and liver oil gave completely negative results when tested for vitamin A.

SUMMARY.

Adult albino rats, previously fed upon diets containing lavish amounts of carotene, were exposed to diets deficient in the vitamin B complex until severe emaciation had resulted. In spite of the disappearance of the fat reserves throughout the body this treatment did not lead to any material diminution in the vitamin A reserves of the liver, which remained at an extremely high level.

Our thanks are due to Dr L. J. Harris for his valuable criticism. The care of the experimental animals was in the capable hands of Mr A. Ward.

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CIII. THE KINETICS OF PEROXIDASE ACTION.

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This work was started with two objects in view. First to test the suggestion of Haldane [1930], that, at low concentrations of hydrogen peroxide, peroxidase would have a temperature coefficient of unity, so that the reaction velocity would be independent of temperature, and secondly to investigate the effect of changes in the concentrations of the hydrogen peroxide and the reducing substrates on the velocity of reaction.

Willstätter and Weber [1926, 1] have shown that the temperature coefficient of peroxidase acting on leucomalachite green is $Q_{10} = 2{\cdot}0$ approximately, over a range of temperature from 0 to 25°. The concentration of hydrogen peroxide was not stated but was probably 2·5 mg. per litre. Now the primary factor in the velocity of enzyme action is presumably the rate of breakdown of the enzyme-substrate compound, but Haldane suggested from kinetic considerations, that at very low hydrogen peroxide concentrations the rate of peroxidase action might rather depend on the number of collisions of hydrogen peroxide molecules with the enzyme, since most collisions would be fruitful. Since this factor is only slightly influenced by temperature, under such conditions peroxidase should have a Q_{10} of unity.

It has long been known that the velocity of peroxidase action increases with increase of peroxide concentration until an optimum is reached, after which further increase in peroxide concentration causes inhibition of the reaction. Willstätter and Weber [1926, 2] found that the inhibition thus produced by excess hydrogen peroxide was combated by increase in the concentration of the reducing substrate, such as pyrogallol or leucomalachite green. This effect has been studied in the second part of this work, using guaiacol and leucomalachite green as the reducing substrates, and from a consideration of the results obtained a new hypothesis is suggested to account for the phenomenon.

Preparation of the enzyme.

The peroxidase was prepared according to the method of Willstätter and Stoll [1918]. Horseradish was used as the source of the enzyme, owing to the absence of aerobic oxidases other than peroxidase.

2·5 kg. of horseradish was cut into slices about 1 mm. thick, and dialysed in running tap-water for 6 days. The peroxidase was then precipitated on the

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plant tissue by treatment with oxalic acid, and the slices were minced to a porridge-like mass from which the enzyme was liberated with baryta. The active extract of $1\frac{1}{4}$ litres was freed from barium by passing carbon dioxide into the solution, and a large amount of protein was precipitated by the addition of 0·9 vol. of alcohol. The clear filtrate was evaporated under reduced pressure to a volume of 30 cc. and, after removal of barium carbonate by filtration, the peroxidase was precipitated in the form of yellow flakes by the addition of 5 vols. of alcohol. The precipitate was centrifuged, washed with alcohol and dried. The product was a yellow powder 0·9 g. in weight. In this form the enzyme was quite stable, but in aqueous solution it lost 5–10 % of its activity in 24 hours at room temperature.

The activity of the preparation.

The activity of the preparation was estimated by determining the purpurogallin number, which is defined by Willstätter and Pollinger [1923] as the mg. of purpurogallin which 1 mg. of enzyme preparation will form in 5 minutes from 5 mg. of pyrogallol and 50 mg. of hydrogen peroxide dissolved in 2 litres of water at 20°, when the amount of enzyme is so small that the change in substrate concentration during the reaction time is practically negligible.

The reaction was stopped at the end of 5 minutes by the addition of dilute sulphuric acid, and the purpurogallin was extracted with ether, the ethereal extracts being made up to a total volume of 500 cc. The amount of purpurogallin present was then estimated in a colorimeter, the standard for comparison consisting of a solution of purpurogallin containing 100 mg. of purpurogallin in a litre of ether. The purpurogallin for the standard was prepared by the method of Perkin and Steven [1903], and was recrystallised from alcohol and ether.

As the result of a large number of estimations an average purpurogallin number of 70 was obtained, compared with an average value of 145 obtained by Willstätter and Pollinger for preparations at the same stage in the purification.

The temperature coefficient.

As previously stated, Haldane [1930] suggested that at sufficiently low concentrations of hydrogen peroxide peroxidase would have a temperature coefficient of unity. The hypothesis depends on the fact that peroxidase is capable of activating many times its weight of hydrogen peroxide per second. Thus Willstätter and Pollinger's best peroxidase preparation could activate 1000 times its weight of hydrogen peroxide per second at 20° when used on leucomalachite green, which is oxidised 120 times as fast as pyrogallol. The hydrogen peroxide concentration was 2·5 mg. per litre (Willstätter and Weber, 1926, 1].

It is extremely desirable when comparing the reaction velocities of peroxidase at different temperatures to take the necessary readings while the

reaction is following a linear course. Hence the change in substrate concentration during the reaction time must be negligible. Now with the purpurogallin method concentrations of the dye of less than 1 mg./100 cc. cannot be measured, and when the hydrogen peroxide concentration is extremely small all the latter may be used up before the necessary amount of purpurogallin is obtained. Thus the method is unsuitable for measurement of the temperature coefficient of peroxidase at low concentrations of hydrogen peroxide. The colour intensity of the triphenylmethane dyes is much greater than that of purpurogallin, and a method using leucomalachite green as acceptor has been found very reliable by Willstätter and Weber [1926, 1]. The procedure is much simpler than that of the purpurogallin method, as it is unnecessary to extract the dye with ether, the amount of malachite green which is formed by the action of the enzyme on the leuco-base being estimated colorimetrically in the reaction mixture itself. The chief disadvantage of the method is the insolubility of the leuco-base, which makes it impossible to work at a reaction much less acid than p_H 4.

The leucomalachite green was prepared by the condensation of benzaldehyde and dimethylaniline in presence of anhydrous zinc chloride, and was repeatedly recrystallised from alcohol and ether, the final product being quite white. A stock solution of reducing substrate was prepared by saturating $N/20$ acetic acid with the leuco-base *in vacuo*. The resulting solution contained approximately 10 mg. of leucomalachite green per 100 cc., and remained colourless for several weeks if kept *in vacuo*. The reaction mixture was made up of 10 cc. of this solution, 0·2 cc. of 0·166 N sodium acetate which adjusted the reaction to p_H 4, the required amount of hydrogen peroxide, 1 or 2 cc. of the enzyme solution prepared by dissolving a few mg. of the powder in 100 cc. of water, and sufficient distilled water to make the volume up to 15 cc. The reaction was carried out in a water-bath at the required temperature for a time varying between 2 and 8 minutes, at the conclusion of which the enzyme was destroyed by the addition of 1 cc. of N sulphuric acid, which after 30 seconds was neutralised with an equivalent amount of sodium carbonate solution. The mixture was shaken to remove carbon dioxide, and the malachite green produced by the reaction estimated in a colorimeter against a standard solution prepared by dissolving 10 mg. of malachite green in a litre of $N/20$ acetic acid. The acetic acid inhibits the hydrolysis of the dye and thus prevents fading of the standard solution.

The range of hydrogen peroxide concentrations which could be used while allowing the experimental readings to be made during the linear course of the reaction was first determined. Fig. 1 illustrates the course of the reaction in presence of different concentrations of hydrogen peroxide at p_H 4. The amount of malachite green formed is plotted against the time. In this case the curves were obtained by placing the reaction solution in the colorimeter cup immediately after the addition of the enzyme, and observing the depth of colour at various times, and thus a complete curve could be drawn for one

solution. The reaction is comparatively slow at a concentration of hydrogen peroxide of 6×10^{-3} M, reaches a maximum at 6×10^{-5} M, and there is little change in velocity between concentrations of 6×10^{-4} M and 6×10^{-6} M, after which the velocity falls off rapidly as the hydrogen peroxide concentration is further decreased. The curves are all linear except in the case of

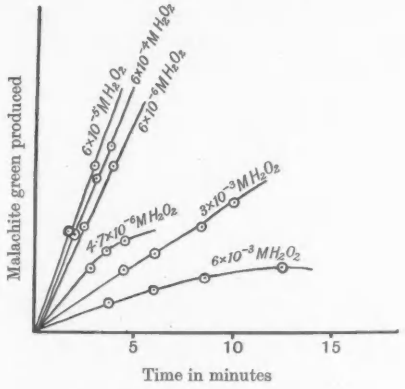


Fig. 1.

the highest concentration where the slight falling off in velocity is probably due to enzyme destruction caused by the high concentration of hydrogen peroxide, and in the lowest concentration where the falling off is due to the using up of a large proportion of the hydrogen peroxide during the course of the reaction.

Having thus found the range of hydrogen peroxide concentrations which it was practicable to use, a number of reaction solutions were made up containing different amounts of peroxide, placed in a water-bath at 20°, and after a definite time the malachite green produced was estimated according to the method already described. The p_s curve was obtained by plotting the reaction velocity against the logarithm of the substrate concentration. The result is shown in Fig. 2. It will be observed that the curve is approximately symmetrical. A possible explanation of its form is suggested in the second part of this work. An advantage of plotting the substrate concentration logarithmically is that the points corresponding to low concentrations which are important in determining the Michaelis constant K_m, that is, the substrate concentration at which half the maximum velocity is reached, are not crowded together. From the curve the value of the apparent affinity of the enzyme for hydrogen peroxide may be obtained, the Michaelis constant, which is the reciprocal of the affinity, being 5×10^{-6} M, which is in agreement with the value of 6×10^{-6} M, calculated by Haldane from the data of Willstätter and Weber [1926, 1].

Willstätter and Weber showed that the Q_{10} of peroxidase at a concentration of peroxide of 25 mg. per litre ($7{\cdot}4 \times 10^{-4}$ *M*) was approximately 2. In order to test the hypothesis of Haldane it was decided to see if this was still the case at a concentration of approximately 10^{-6} *M*. At this concentration it was found impossible to obtain readings while the reaction was following

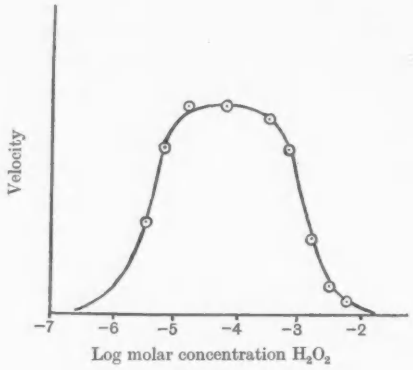


Fig. 2.

a linear course, using the original method of estimation, and to make this possible the method of estimation was altered. 100 cc. of the leucomalachite green solution were used instead of 10 cc., and the malachite green was extracted twice with amyl alcohol, the combined extracts being made up to a total volume of 10 or 15 cc. By this means the dye intensity could be increased 10-fold. The standard solution for comparison in the colorimeter was obtained by extracting the original standard with an equal volume of amyl alcohol.

By means of this method curves were obtained at a concentration of hydrogen peroxide of $1{\cdot}3 \times 10^{-6}$ *M* showing the relative velocities of the

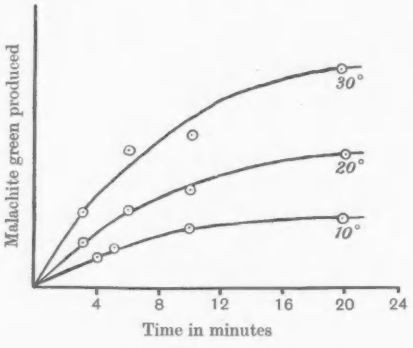


Fig. 3.

reaction at temperatures of 10°, 20° and 30°. It will be observed (Fig. 3) that the reaction follows a linear course for a considerable period, and thus it is possible to obtain the initial velocities at the three temperatures without drawing the tangents to the curves. The curves give Q_{10} 1·9 between 10° and 20°, and 1·8 between 20° and 30°, and thus, contrary to the suggestion of Haldane, the temperature coefficient appears to have been little affected by the change in hydrogen peroxide concentration.

The question was further studied by comparing the p_s curves obtained at different temperatures. If the temperature coefficient is different for different hydrogen peroxide concentrations, one would expect an alteration in the shape of the p_s curve on the horizontal as well as the vertical scale, but, on the other hand, if the temperature coefficient is independent of hydrogen peroxide concentration, the only effect of increase of temperature should be to increase the vertical without altering the horizontal scale. p_s curves were therefore obtained at temperatures of 0°, 20° and 30°, and are shown in Fig. 4. The

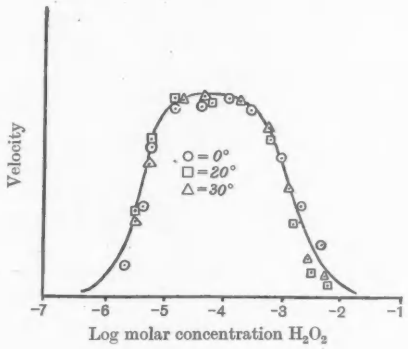


Fig. 4.

curves have all been reduced to the same vertical scale the better to illustrate the small change in affinity. It is obvious that there is little change in the horizontal range of the curves and thus one would expect the temperature coefficient to remain at almost the same figure even at still lower peroxide concentrations than those employed.

Dann [1931], working with citric acid dehydrogenase, has successfully used the temperature coefficient of the affinity as a means of estimating the heat of formation of the enzyme substrate compound. From the curves of Fig. 4 it is obvious that the temperature coefficient of the affinity of peroxidase for hydrogen peroxide is too small to be accurately measured, and thus the heat of formation of the enzyme-substrate compound must be very small.

Haldane's prediction was based upon the assumption that the gas laws could be applied to a substance dissolved in a liquid, so that the frequency of collisions could be approximately deduced by regarding the substance as

in the gaseous state under a pressure equal to its osmotic pressure in solution. The results of this work do not bear out the assumption and may be regarded as evidence that the frequency of collisions in the liquid state is at least several hundred times as great as that indicated by an application of the ordinary gas laws.

The object of the second part of this work was to investigate the relations between the hydrogen peroxide and the reducing substrates at the enzyme. Many years ago Bach [1904], working on the oxidation of hydriodic acid by peroxidase, showed that excess of hydrogen peroxide inhibited the reaction. He attributed the inhibition to irreversible destruction of the enzyme. Willstätter and Weber [1926, 2] investigated this inhibition, and showed that while irreversible destruction of the enzyme may be caused by high concentrations of hydrogen peroxide, reversible inactivation is also produced. In such cases the velocity may be increased either by increasing the concentration of reducing substrate (pyrogallol or leucomalachite green), or by removing some of the hydrogen peroxide by means of catalase. They suggest as an explanation of the reversible inhibition that the peroxide is able to combine with the enzyme in either of two ways, giving an active and an inactive enzyme-substrate compound respectively. Thus in presence of low concentrations of hydrogen peroxide the peroxide combines with the enzyme in the form $\begin{matrix}H\\H\end{matrix}\!\!>\!O = O$ to give an unstable compound which is catalytically active. In high concentrations, however, it combines as H—O—O—H, giving a relatively stable enzyme-substrate compound which is catalytically inactive.

This explanation is unsatisfactory, since by the law of mass action the ratio of the two forms of the enzyme-substrate compound should be independent of the substrate concentration. On the basis of the results obtained by the author an alternative hypothesis is put forward. This was suggested by the addition compound theory of enzyme action [Woolf, 1931], according to which for an enzyme reaction to occur all the substrates must be combined at the enzyme. The explanation depends on the assumption that the reducing substrate combines with the enzyme, a view which has hitherto not been accepted. Haldane [1930] summing up the evidence on this question writes "The reducing substrates of peroxidase appear to act, not by combining with it as a preliminary to oxidation, but by combating the inhibition of its activity by the relatively high hydrogen peroxide concentrations used." The results obtained, however, indicate that the reducing substrate does combine with the enzyme. It is further postulated that the hydrogen peroxide combines with the enzyme at a group which is moderately specific, as it can only be replaced by compounds of related structure such as ethyl hydrogen peroxide [Wieland and Sutter, 1930]. The group with which the reducing substrate combines has a far wider range of specificity, as shown by the variety of substances which can be oxidised by the peroxidase system. It is suggested that hydrogen peroxide is also capable of combining at the latter group. When

both peroxide and reducing substrate are combined at the enzyme at their respective groups, an active enzyme-substrate compound is produced, but in presence of excess of hydrogen peroxide the latter may combine with the enzyme at the non-specific group, thus keeping the reducing substrate from the enzyme and causing inhibition. Such inhibition would be reversible and could be combated by either of the methods described by Willstätter and Weber, *i.e.* by raising the concentration of reducing substrate or by removing some of the hydrogen peroxide with catalase. In both these cases the reducing substrate would be in a better position to compete with the peroxide for the enzyme and thus the reaction velocity would be increased.

The relations between the hydrogen peroxide and the reducing substrate at the enzyme were first studied by obtaining a series of p_s curves each at a different concentration of leuco-malachite green. Now at p_H 4 a saturated solution of leucomalachite green contains only 10 mg. of the base in 100 cc., and thus it is impossible to obtain the wide range of concentrations of reducing substrate which is desirable. At more acid reactions, however, the solubility is considerably increased, being about 10 times as great at p_H 3·2, at which reaction the work to be described was carried out. The leucomalachite green was dissolved in *N* acetic acid, and the reaction was adjusted to p_H 3·2 with sodium acetate.

Fig. 5 shows the series of peroxide p_s curves obtained in presence of

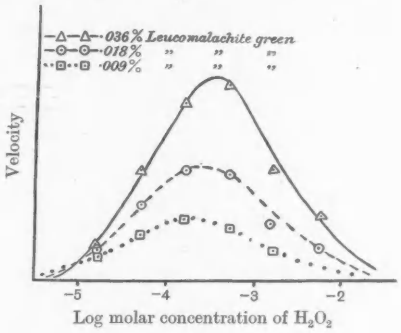


Fig. 5.

different concentrations of leucomalachite green. In the case of a simple enzymic reaction, the curve obtained by plotting the substrate concentration is a rectangular hyperbola or Michaelis curve, or if the substrate concentration is plotted logarithmically an S-shaped curve results which is of the same form as when the amount of dissociation of a weak acid is plotted against p_H. In the case of peroxidase a similar curve would be expected if there were no competition between the two substrates. It will be observed that the p_s curves obtained with peroxidase, shown in Fig. 5, do not resemble the typical form of p_s curve just described. They are symmetrical curves similar to those

obtained when the dissociation residues of ampholytes are plotted against p_H. As the peroxide concentration is increased the velocity rises rapidly until an optimum is reached, after which further increase in concentration causes a rapid fall in reaction velocity. It is suggested that this fall in velocity is due to the competitive combination of hydrogen peroxide at the non-specific grouping of the enzyme. This view is further supported by consideration of the effect of increasing the concentration of reducing substrate. As the concentration of reducing substrate is increased the range of peroxide concentration over which activity is obtained is extended, the system being catalytically active in higher concentrations of peroxide. The curves obtained, however, are still approximately symmetrical, as the position of the optimum is correspondingly shifted. Besides the extension of the horizontal range of the curves, the vertical range is also extended by increase in the leucomalachite green concentration. The increase in reducing substrate concentration has a marked activating effect on the velocity when the peroxide is present in such concentration as formerly produced inhibition. These facts are in complete accordance with the hypothesis outlined above.

Leucomalachite green was used as the reducing substrate in the work on the temperature coefficient because, owing to its colour intensity, the dye produced could be estimated in very low concentrations. For the present work, however, its insolubility rendered it unsuitable. It was therefore decided that further work on the subject should be done with another more soluble substrate. Nitrite, which is oxidised by the peroxidase system to nitrate, and quinol, which is oxidised to quinone, were both tried, but had to be discarded owing to the difficulties of accurately estimating, in presence of hydrogen peroxide, the small amounts of nitrate and quinone produced.

The reducing substrate finally chosen was guaiacol, which, according to Bertrand [1903], is oxidised to a complex red compound, tetraguaiacol.

$$4C_6H_4OH.OCH_3 + 2O_2 = 4H_2O + \begin{matrix} C_6H_3(OCH_3)—O—O—C_6H_3(OCH_3) \\ | \qquad\qquad\qquad\qquad | \\ C_6H_3(OCH_3)—O—O—C_6H_3(OCH_3) \end{matrix}$$

The method of estimation employed was that of Bansi and Ucko [1926]. The method is a colorimetric one, essentially similar to the leucomalachite green method, but the standard solution for comparison in the colorimeter is an artificial one, made by mixing cobalt nitrate solution and potassium dichromate solution until the correct tint is obtained. The reaction is stopped by the addition not of acid, which causes the colour to fade rapidly, but of mercuric chloride solution.

Employing this method, the series of peroxide p_s curves shown in Fig. 6 was obtained at p_H 4·7, using four different concentrations of guaiacol. The curves are similar to those obtained with leucomalachite green (Fig. 5), being symmetrical and resembling in form the dissociation residue curves of ampholytes. Increase in guaiacol concentration has the same effect as increase in leucomalachite green concentration, the curves being extended in range both

horizontally and vertically. The increase of guaiacol concentration shows an activating effect on the velocity when the peroxide is present in concentration so high as formerly to have produced an inhibition. This was also the case with leucomalachite green. These facts afford additional support for the

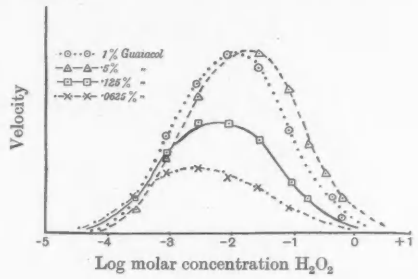


Fig. 6.

theory that the peroxide and reducing substrate compete for the relatively non-specific group of the enzyme. It will be observed that with the highest concentration of guaiacol the velocity at low concentrations of peroxide is less than that when the concentration of guaiacol is 0·5 %, though in higher concentrations of peroxide an activation is produced. This was not observed with leuco-malachite green, possibly because, owing to the insolubility of the latter compound, the necessary concentration to produce the effect could not be obtained.

This phenomenon was further studied in a different way. A series of curves was obtained at p_H 4·7 showing the relation between the concentration of guaiacol and the initial reaction velocity, in presence of several constant concentrations of peroxide. These are shown in Fig. 7. A true Michaelis curve

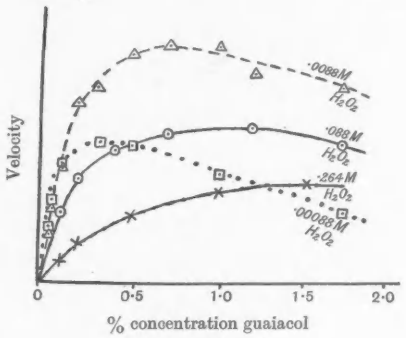


Fig. 7.

is not obtained. At the highest peroxide concentration employed, 0·264 *M*, the optimum concentration of guaiacol has not been reached, the reaction

velocity still increasing with increase in guaiacol concentration. At a concentration of 0·088 *M* peroxide, an optimum guaiacol concentration can be reached, but as the guaiacol is further increased, the reaction velocity, instead of remaining constant, begins to decrease. This fall in velocity is better seen in the other two curves, in presence of still lower peroxide concentrations. As the concentration of peroxide is decreased the optimum velocity is obtained at progressively lower guaiacol concentrations. These curves are shown plotted logarithmically in Fig. 8. They are approximately symmetrical, and again resemble dissociation residue curves of ampholytes.

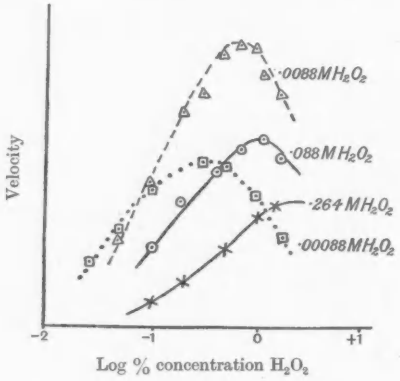


Fig. 8.

The curves of Figs. 7 and 8 cannot be completely explained by the simple theory that the peroxide competes with the reducing substrate at the enzyme, and some extension of the theory is accordingly necessitated. The form of the curves does not seem to support the possibility that the reducing substrate competes with the peroxide at what has been assumed to be the specific combining group of the enzyme. A more adequate explanation which fits the curves, at any rate qualitatively, is to be found from a study of the following considerations.

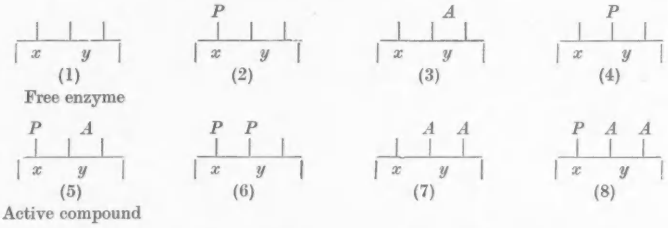
Simple enzyme p_s curves are S-shaped, *e.g.* that of saccharase, further increase in substrate concentration after the maximum velocity has been reached having little effect on the velocity until, in the case of saccharase, the substrate concentration is so high that water becomes a limiting factor [Nelson and Schubert, 1928]. In the case of other enzymes however, and in particular of lipase, after the optimum velocity is reached, further increase in substrate concentration causes a rapid fall in the velocity of the reaction, approximately symmetrical curves being obtained, as was observed, *e.g.* by Bamann [1929], for the velocity of hydrolysis of ethyl *d*-mandelate by dog-liver lipase. Haldane [1930] has suggested that such a phenomenon may be explained by the assumption that the substrate is capable of combining

twice with the enzyme, giving an inactive compound. Whereas in the case of the normal enzyme-substrate compound ES, the enzyme is united to the ester by two linkages, one at the alcohol, the other at the acid residue, in the case of the ES_2 compound each substrate molecule is only once united, and hence the strain producing hydrolysis does not arise. This is illustrated by the diagrams shown below which are taken from a paper on lipase by Murray [1930].

Murray tested the suggestion of Haldane using the esterase of sheep-liver, and found that it provided a satisfactory explanation of his experimental results.

Such a hypothesis affords an adequate explanation of the action of excess of reducing substrate in producing inhibition of peroxidase action. It is therefore suggested that each molecule of reducing substrate normally combines at two linkages with the enzyme, forming a compound which, when combined with peroxide at the requisite group, gives a catalytically active enzyme-substrate compound. The reducing substrate is, however, also capable of combining in a different way with the enzyme, forming an inactive compound ES_2, a molecule combining with each of the two linkages across which one molecule normally combines. In presence of excess of reducing substrate this inactive compound is naturally produced to a large extent and thus inhibition of the reaction is caused.

On this hypothesis, in a solution of peroxidase, peroxide, and reducing substrate at least 8 forms of the enzyme may be present. These are shown in the scheme below, in which the combining groups of the enzyme are denoted by x (the group specific for peroxide), and y, the peroxide by P, and the reducing substrate or acceptor by A.



The active enzyme-substrate compound is denoted by 5. In presence of excess of hydrogen peroxide, a large amount of the inactive compounds 4 and 6 will be produced, and thus inhibition will be caused. In presence of excess of reducing substrate a large proportion of the enzyme will be combined to form the compounds 7 and 8, which are also inactive and produce inhibition. This appears to be the simplest explanation of the results obtained.

SUMMARY.

The suggestion of Haldane [1930] that the temperature coefficient of peroxidase action would be approximately unity at very low concentrations of hydrogen peroxide has been tested and found to be erroneous. The temperature coefficient is just under 2 at all hydrogen peroxide concentrations.

The effect has been studied of variation of the concentrations of hydrogen peroxide and the reducing substrate on the velocity of peroxidase action, the reducing substrates used being leucomalachite green and guaiacol. The results suggest that both peroxide and reducing substrate must be combined at the enzyme before catalysis can take place.

I wish to thank Prof. J. B. S. Haldane and Dr B. Woolf for much help and advice, and Sir F. G. Hopkins for his interest and encouragement during the course of this work. My thanks are also due to the Department of Scientific and Industrial Research for a part-time grant.

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CIV. INVESTIGATIONS ON THE PREPARATION AND BEHAVIOUR OF VITAMIN B_1 CONCENTRATES FROM YEAST.

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Introduction.

In a previous publication a method was described for the concentration of vitamin B_1 from wheat embryo, which had been attended with a considerable measure of success [Guha and Drummond, 1929]. A semi-crystalline preparation was obtained, of which the pigeon-curative day-dose was found to be of the order of 0·005 mg. It also promoted good growth in young rats, placed on a vitamin B_1-deficient diet, in a daily dose of 0·015 mg. The preparation, however, was obviously impure, and when subjected to fractionation by gold chloride behaved as if the vitamin had been split into two fractions, as a mixture of the substances obtained from the gold precipitate and filtrate appeared to be more active than either. As, however, a very small amount of material was at our disposal, the number of pigeon and rat tests was very inadequate, and, therefore, our results had to be taken with reserve. Recently, however, Reader [1930] has stated that the experimental "beriberi" condition in rats is a complex syndrome, resulting from a deficiency of two factors, B_1 and B_4. Furthermore, the crystals obtained by Jansen and Donath [1926] from rice polishings, which were originally claimed to be vitamin B_1 hydrochloride, have also been stated to contain vitamin B_4 [Jansen, Kinnersley, Peters and Reader, 1930]. Further information on this subject will be awaited with interest.

The present investigation was undertaken with the double object of finding out how far the method of fractionation, which had been previously applied to wheat embryo, could be successfully employed for the concentration of the vitamin from yeast, and whether any evidence about the multiple nature of vitamin B_1 could be obtained when yeast was used in place of wheat embryo as the raw material. Brewery bottom yeast was employed by Seidell [1926] for the concentration of vitamin B_1, but as his method of biological assay depended on the prevention of loss of weight of pigeons on a polished rice diet, it is clear from recent work on pigeons that the tests were complicated by other factors besides B_1 [Williams and Waterman, 1928; Carter, Kinnersley

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and Peters, 1930, 1, 2; Guha, 1929]. Baker's yeast has usually been employed by Peters and his co-workers and he has recently published improvements in his technique by fractional precipitation by means of phosphotungstic acid at different hydrogen ion concentrations [Kinnersley and Peters, 1930].

The fractionation recorded in the present paper was carried out with brewer's top yeast, as its aqueous extract was found by means of preliminary experiments, based on rat-growth tests, to be somewhat richer in vitamin B_1 than an aqueous extract of baker's yeast. The method has been standardised up to the platinum stage by several repetitions. The preparation obtained at this stage promotes good growth in young rats in a daily dose of 0·075–0·10 mg. It is expected that the material collected by this method of fractionation in several operations might be further fractionated with advantage. Incidentally the relation, if any, between vitamin B_1 on the one hand and secretin, co-zymase and bios on the other has been investigated. The absorption spectra and certain other properties of B_1 concentrates are also described.

Experimental.

Biological technique.

The method of biological assay described before [Guha and Drummond, 1929] has been used in these experiments with a few modifications. Young rats, about 50 g. in weight, were fed on a basal diet of the following composition:

Rice starch	65 %
"Light white casein" (B.D.H.)	21 %
Palm kernel oil	10 %
Salt mixture (Osborne and Mendel)	4 %

This diet was supplemented daily with 2 drops of cod-liver oil and 1·5 cc. of a 50 % solution of autoclaved marmite in order to supply vitamins A, D and B_2. The latter preparation was obtained by mixing the commercial yeast extract, marmite, with baryta, bringing the mixture roughly to p_H 9 and autoclaving for 1½ hours at 124°, barium being finally removed by means of sulphuric acid. Numerous experiments have proved that such a preparation contains large quantities of vitamin B_2 and negligible quantities of B_1. When the weight of the young animals began to decline on this diet they were fed with the vitamin B_1 fraction to be tested, and the standard of growth was taken to be at least 10–12 g. per week. Each fraction was tested on at least 2 rats, usually more. Rat-growth tests were adopted as a matter of routine, while these were periodically checked by pigeon-curative tests. The pigeon-curative day-dose was computed by dividing the amount injected by the number of days for which protection was afforded.

The technique of assaying vitamin B_1 has recently been discussed by several investigators [Smith, 1930; Kinnersley, Peters and Reader, 1930].

Smith describes an apparently promising method, based on curative tests with rats, by which he seems to have obtained very regular results. In our experience, rat-growth tests have yielded fairly uniform results. Fig. 1 illustrates the fact that under strictly controlled conditions it is possible to get

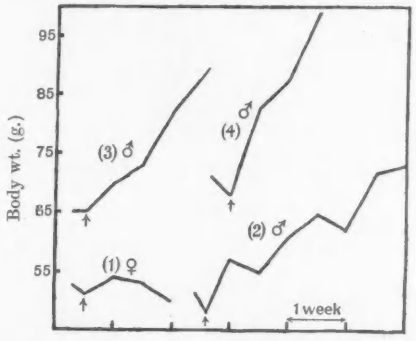

Fig. 1. Experiments with an aqueous-alcoholic extract of brewer's yeast.
Curve (1). ↑ dose = 0·15 cc. Curve (2). ↑ dose = 0·225 cc.
„ (3). ↑ dose = 0·33 cc. „ (4). ↑ dose = 0·45 cc.

approximately proportionate growth responses to graded doses of a given vitamin B_1 preparation. We have adhered to this method in order to have results comparable with those obtained from wheat germ. It is possible, however, that at the last stages of fractionation, Smith's curative method might give more trustworthy results. It would, doubtless, be more economical.

Tests with known substances.

In the earlier paper, it was stated that various substances like nucleic acid, betaine, nicotinic acid, which had at one time or other been credited with properties of B_1, were found to be inactive by the rat-growth tests. The following substances have now been fed in the doses stated, again with negative results.

Substance tested	Dose (mg.)
Adenine picrate	2 and 4
Guanine hydrochloride	2 „ 4
Uracil	2 „ 4
Synthetic *dl*-thyroxine	0·5 „ 1
Histamine acid phosphate	0·1 „ 0·2
"Adrenalinum" (B.D.H.) (faintly acidified with HCl) ...	0·1 „ 0·2

The relative vitamin B_1 values of extracts of brewer's top yeast and baker's yeast.

Aqueous extracts of brewer's top yeast and baker's yeast, made by extraction with boiling water and boiling for 3 minutes were, compared for vitamin B_1 activity. The brewer's yeast gave somewhat more active extracts

in most cases. One sample of baker's yeast was, however, found particularly rich in vitamin B_1 (Fig. 2). It is probable that there is a considerable variation

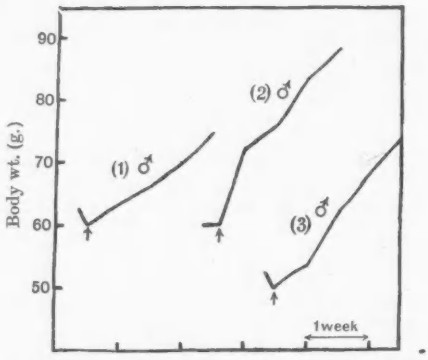

Fig. 2. Tests with aqueous extracts of brewer's top yeast and baker's yeast.
Curve (1). ↑ dose≡0·5 g. baker's yeast.
„ (2). ↑ dose≡0·5 g. brewer's top yeast.
„ (3) illustrates the potency of a particularly active variety of baker's yeast; ↑ dose≡0·5 g. yeast.

in the vitamin B_1 potency of different samples of both varieties of yeast [see also Quinn, Whalen and Hartley, 1930]. Pirie's method of extraction of yeast for glutathione [Pirie, 1930] also extracts vitamin B_1 effectively from both brewer's and baker's yeast.

Procedure of fractionation.

Brewer's yeast was employed for the fractionation of vitamin B_1 recorded in this paper. This method has met with success up to the platinum stage every time it was tried, though, unfortunately, the same degree of concentration was not achieved at this stage as had been obtained with wheat germ [Guha and Drummond, 1929]. The following account represents a fairly standardised method.

Stage 1. Extraction with aqueous alcohol. 3178 g. of fresh brewer's top yeast were extracted with 2·5 l. of 95 % alcohol at 60–70° for 4–6 hours. The residue, after filtration, was re-extracted with 1·75 l. of 50 % alcohol at the same temperature for the same period. After concentration under reduced pressure, the first extract was active in a daily dose, representing 0·64 g. of the fresh undried yeast, which contained 40 mg. total solids, of which 33·5 mg. were organic. The active dose of the second extract was equivalent to 1·6 g. of the fresh yeast and carried 25 mg. of the total solids, of which 22·4 mg. were organic (Fig. 3).

Stage 2. Fractionation by lead acetate. The two extracts of the preceding stage were combined and precipitated with 120–150 g. of neutral lead acetate in aqueous solution. The filtrate from the lead precipitate was freed from lead

by hydrogen sulphide in a slightly acid medium, the hydrogen sulphide being later removed by concentration *in vacuo* to a volume of 1500 cc. This material was active in daily doses containing 40 mg. of total solids, of which 30 mg. were organic, equivalent to 0·75 g. of the fresh yeast (Fig. 3).

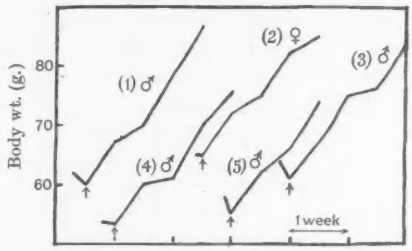

Fig. 3.

Curve (1). The 95 % alcoholic extract of brewer's yeast; ↑ dose≡0·64 g. of fresh yeast.
„ (2). The second 50 % alcoholic extract of brewer's yeast; ↑ dose≡1·6 g. of fresh yeast.
„ (3). The lead filtrate; ↑ dose≡0·75 g. yeast.
„ (4). The fuller's earth extract; ↑ dose≡1·38 g. yeast.
„ (5). Silver nitrate-baryta fraction at p_H 6·8; ↑ dose≡1·5 g. yeast.

Pigeon-curative tests with 4 birds showed that the average day-dose contained 12 mg. total solids of which 9·3 mg. were organic.

Stage 3. Adsorption by fuller's earth. The lead filtrate was brought, if necessary, to p_H 4·5 and agitated for 20 minutes with 50 g. fuller's earth[1] and left overnight. The supernatant fluid was syphoned off and the bottom layer sucked dry. The fuller's earth residue, which weighed 67 g., was extracted by grinding up with 200 cc. of saturated baryta and immediately filtering with strong suction. The total operation of grinding and filtering took 5–7 minutes. The filtrate was received in a slight excess of concentrated sulphuric acid. The fuller's earth residue was washed with a little baryta and then with water. After the removal of the precipitated barium sulphate from the filtrate, the fraction proved to be active in doses containing 1·4 mg. total solids, of which 1·3 mg. were organic. The dose was equivalent to 1·38 g. yeast (Fig. 3). Curative tests with 3 pigeons showed that the average day-dose contained 0·57 mg. total solids of which 0·53 mg. was organic.

Stage 4. Fractionation by silver nitrate and baryta. The above fuller's earth extract, which was acid to Congo red, was treated with 15 cc. of 50 % silver nitrate solution, which represented an excess, and then treated with the requisite quantity of saturated baryta to bring the p_H to 4·5 and allowed to settle for 2 hours. The precipitate which was very slightly active was discarded

[1] In our experience different samples of fuller's earth have been found to differ greatly in their adsorbing capacity. It is therefore desirable to stock a quantity of fuller's earth, after its suitability has been established. In these experiments Boots's fuller's earth was used. Experiments with the "acid clay" used by Jansen and Donath show that it is also a very efficient adsorbent for the vitamin.

and the filtrate brought to p_H 6·8 by adding the requisite amount of saturated baryta. After being allowed to stand overnight, the brown precipitate was filtered off (wet weight 5 g.) and decomposed by hydrochloric acid. On filtration, a straw-coloured filtrate was obtained, of which the rat day-dose contained 0·345 mg. total solids, of which 0·29 mg. was organic matter (Fig. 3). This was equivalent to 1·5 g. yeast. Further fractionation of the main filtrate at p_H 8 yielded negative results. Curative tests on 2 pigeons with the silver fraction precipitated at p_H 6·8 showed that the average day-dose contained 0·16 mg. total solids of which 0·13 mg. was organic.

Stage 5. Fractionation by phosphotungstic acid. The fraction obtained at p_H 6·8, which is thus seen to contain almost all the activity, had a volume of 200 cc., and this was treated with the required quantity of concentrated H_2SO_4 to make roughly 5 % acid, and then with 14 cc. of a saturated solution of phosphotungstic acid in 5 % H_2SO_4, which represented a slight excess. The precipitate was removed after 24 hours, filtered under strong suction (dry weight = 3·5 g.), dissolved in 70 cc. of 50 % acetone and filtered from a very small amount of undissolved residue. The clear red-brown filtrate was treated gradually with 50 cc. of 5 % H_2SO_4 with constant stirring. At this stage a slight turbidity remained, which did not disappear on warming to 60°. A gummy material separated, which, on leaving the mixture for a week in the refrigerator, turned granular. This orange-yellow precipitate (*a*) was filtered, sucked dry (weight = 1 g.) and decomposed by dissolving in 50 cc. of 50 % acetone and shaking up with 30–40 cc. baryta, which were sufficient to make the solution alkaline to phenolphthalein. The precipitate of barium phosphotungstate was filtered off immediately and the filtrate acidified with sulphuric acid. After filtration from $BaSO_4$ and the removal of acetone *in vacuo* the clear pale straw-coloured filtrate proved to be active in a daily dose containing 0·29 mg. total solids, equivalent to 1·6 g. yeast (Fig. 4)[1]. The filtrate obtained after removal of precipitate (*a*) on treatment with an excess (300 cc.) of 5 % H_2SO_4 yielded a further quantity of precipitate, which was found to be inactive.

The filtrate obtained after the removal of the original phosphotungstic precipitate, on standing in the refrigerator for several days, deposited a very small amount of colourless crystals which appeared as a homogeneous mass of rhombohedra under the microscope. After decomposition by baryta, it proved to be entirely inactive. On evaporation and examination it was found to be crystalline and homogeneous. In view of the small amount of material, however, its identity was not further investigated.

[1] It is not easy to understand why Kinnersley and Peters [1930] failed to obtain the vitamin in the acetone-soluble phosphotungstic fraction. The phosphotungstates of the vitamin fractions from wheat germ [Guha and Drummond, 1929], from rice polishings [Jansen and Donath, 1926], as also from brewer's yeast (as shown in the present communication) are apparently acetone-soluble. It is possible, however, that this solubility is not a property of the vitamin *per se* but is due to the presence of the acetone-soluble phosphotungstates of other materials, which may not be precipitated under the somewhat altered conditions of Kinnersley and Peters.

The average pigeon-curative day-dose of the active phosphotungstic acid fraction, tested with 3 birds, contained 0·052 mg. total solids.

Stage 6. Extraction with absolute alcohol. The above active phosphotungstic fraction was evaporated to dryness in a vacuum desiccator containing soda lime and was extracted four times with hot (70–80°) absolute alcohol. The pale straw-coloured extract, after filtration, measured 116 cc., and a portion of it, after removal of alcohol, was found to be active in doses containing 0·274 mg. total solids, equivalent to 1·76 g. yeast (Fig. 4).

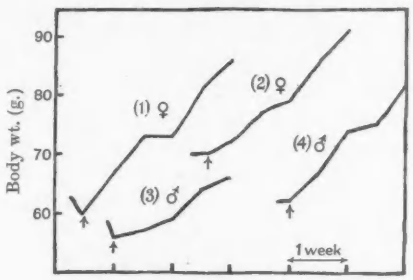

Fig. 4.

Curve (1). Phosphotungstic acid fraction; ↑ dose≡1·6 g. yeast.
„ (2). Alcoholic fraction; ↑ dose≡1·76 g. yeast.
„ (3). Platinum precipitate fraction; ↑ dose≡2·37 g. yeast.
„ (4). Platinum precipitate fraction; ↑ dose≡3·16 g. yeast.

Stage 7. Fractionation by platinic chloride. The alcoholic extract was concentrated in a vacuum desiccator to a small bulk, and, after the removal of a light pale yellow precipitate which had separated, the filtrate (30 cc.) was treated with 6·5 cc. (excess) of a 5 % solution of platinic chloride in absolute alcohol. It rapidly deposited a micro-crystalline orange-yellow precipitate, which was left in the refrigerator overnight. As previous experiments showed that decomposition of the platinum precipitate by hydrogen sulphide frequently results in a considerable loss of activity[1], the platinum precipitate, after being filtered off and washed with a little absolute alcohol, was decomposed by means of potassium chloride by the method of Ewins [1914]. The platinum filtrate was also similarly decomposed. The platinum precipitate was active in doses containing 0·075–0·10 mg. total solids, equivalent to 2·37–3·16 g. yeast (Fig. 4). The platinum filtrate was entirely inactive in doses equivalent to 5 g. yeast. A mixture of half the effective dose of the platinum precipitate and a dose of the platinum filtrate, equivalent to 2·5 g. of original yeast, was almost entirely inactive.

Curative tests with 5 birds showed that the average day-dose of the platinum precipitate fraction contained 0·047 mg. total solids.

[1] In two instances about 80 % of the activity disappeared on treatment of the platinum precipitate with hydrogen sulphide. Such a large loss under the same treatment was not found to occur with wheat germ fractions [*cf.* Guha and Drummond, 1929], though the KCl method is undoubtedly a better one for such purposes.

Stage 8. Fractionation by gold chloride. The solution obtained by the decomposition of the platinum precipitate was evaporated to dryness in a vacuum desiccator. The residue was extracted with 13 cc. of hot absolute alcohol, filtered from a small amount of insoluble residue, and treated with 2 cc. of a 4 % solution of gold chloride in absolute alcohol. As no precipitate was formed, the solution was concentrated to a very small volume (0·5 cc.) in a desiccator, and then filtered from a very minute amount of an orange-yellow semi-crystalline precipitate. The gold precipitate, thus formed, was much more soluble in alcohol than in water.

The gold precipitate was dissolved in 10 cc. of 90 % alcohol and shaken up with precipitated metallic silver, according to the method of Dudley [1929], to precipitate the gold. The gold filtrate was also similarly freed from gold. The above operation, however, resulted in a great loss of activity. The fraction from the gold precipitate produced only slight growth in doses containing 0·054 mg. total solids, equivalent to 6 g. of the original yeast, while the gold filtrate was inactive in the same dose but produced slight growth in doses, containing 0·2 mg. total solids, equivalent to 11·2 g. of yeast. A mixture of 0·0357 mg. of the gold precipitate and 0·1 mg. of the gold filtrate resulted also in very slight growth (Fig. 5).

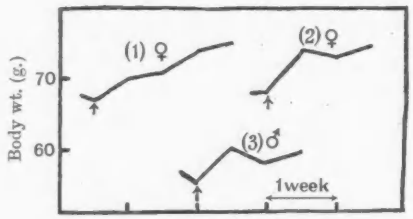

Fig. 5.

Curve (1). Gold precipitate fraction; ↑ dose = 0·054 mg. total solids.
„ (2). Gold filtrate fraction; ↑ dose = 0·2 mg. total solids.
„ (3). ↑ dose = 0·0357 mg. of the gold precipitate fraction *plus* 0·1 mg. of the gold filtrate fraction.

Curiously, though the rat-growth tests gave such unsatisfactory results, the pigeon-curative tests indicated that there was quite considerable activity in both the gold precipitate and filtrate fractions, the former having twice

Gold precipitate fraction

Pigeon no.	Dose injected (mg.)	Days of protection
1	0·05	3
2	0·05	2
3	0·05	6
4	0·05	7

Average day-dose = 0·012 mg.

Gold filtrate fraction

Pigeon no.	Dose injected (mg.)	Days of protection
1	0·1	8
2	0·15	2
3	0·25	8
4	0·3	16

Average day-dose = 0·024 mg.

Gold precipitate fraction (g.p.) *plus* Gold filtrate fraction (g.f.)

Pigeon no.	Dose injected (mg.) g.p.	g.f.	Days of protection
1	0·05	+ 0·05	8
2	0·05	+ 0·05	5
3	0·05	+ 0·05	9

Average day-dose = 0·014 mg.

the activity of the latter. A combination of the two fractions did not show any markedly enhanced effect. These results are shown in the above tables.

These curious results are discussed later.

Notes on the course of fractionation.

(1) The extraction of the brewer's yeast, if carried out simply by boiling with water, also gives satisfactory results and the fractionation can be followed to the platinum stage as described above.

(2) The precipitation by lead acetate at Stage 2 does not effect any considerable concentration because, while some inactive material is removed, a certain amount of loss occurs due, probably, to adsorption. But unless this preliminary purification is carried out, the subsequent fractionation by fuller's earth at Stage 3 does not appear to be so satisfactory. Adsorption by charcoal also ceases to be selective and occurs imperfectly at p_H 3, 4, 5 and 6, unless the lead stage is introduced. Kinnersley and Peters [1928] insert the lead stage before adsorption with charcoal.

(3) Levene and Van der Hoeven [1924] considered that barium ions precipitate a portion at least of the vitamin. Seidell [1926] was, therefore, led to the use of sodium hydroxide instead of saturated baryta for the elution of the vitamin from the "activated" fuller's earth. As it was not desired to introduce soluble inorganic material unnecessarily, the question was reinvestigated. Under identical conditions elutions by equal volumes of $N/8$ NaOH and saturated baryta are equally effective, though one extraction by $N/8$ NaOH is more effective than one by $N/8$ $Ba(OH)_2$. There does not therefore appear to be any ground for assuming that the vitamin forms an insoluble complex with barium ions.

(4) It has been found that kieselguhr at p_H 4·5, can also adsorb the vitamin which can be eluted by saturated baryta. The adsorption by kieselguhr is, however, far less efficient than that by fuller's earth, as only about one-third of the vitamin is adsorbed by the former.

Vitamin B_1 and secretin.

Three different preparations of vitamin B_1, one obtained at Stage 1 by aqueous-alcoholic extraction, another at Stage 2 after lead fractionation and a third prepared by extracting with water, adsorbing with norite charcoal and fractionating with phosphotungstic acid, which were all highly active, were examined for their secretagogue activity. The tests were kindly carried out by Dr Brocklehurst and Mr Cunningham on amytal-anaesthetised cats. The third concentrated preparation did not show any appreciable effect at all, while the first two impure preparations had such a great depressor effect that in one case the animal was almost instantly killed. The nature of this depressor principle is not understood.

These observations confirm the earlier experiments of Anrep and Drummond [1921], as opposed to those of Uhlmann [1918] and of Voegtlin and Myers [1919].

Vitamin B_1 and "bios."

In confirmation of the earlier observations with wheat germ preparations [Guha and Drummond, 1929] it has been found that the "bios" activity of the yeast fraction obtained at Stage 5 is negligible, though this latter fraction was potent for rats in a daily dose of 0·3 mg. It is, however, of interest to point out in this connection that Williams and Roehm [1930] consider that while vitamin B_1 cannot stimulate the growth of yeast by itself, it can do so in conjunction with a material obtained from yeast extracts, which is not adsorbed by fuller's earth. Jansen and Donath's crystals are stated to exhibit this effect in minute amounts. In fact, from a parallel study of the growth of different varieties of yeast, Williams and Bradway [1931] have come to the conclusion that while "bios," as required by Wildier's strain of yeast and presumably by Narayanan's [1930], has not been fractionated, the strain used by the Toronto workers requires multiple factors, of which apparently inositol is one. Williams and Bradway consider that there are four "bios" factors and there might be some relation between vitamin B_1 and one of these. The writer's observations do not cover this point, as the tests were carried out with the same strain of yeast as was used by Narayanan.

Vitamin B_1 and co-zymase.

Kinnersley and Peters [1928] and Euler and Myrbäck [1929] consider that vitamin B_1 and co-zymase are not identical. As the most potent co-zymase preparations have been found to give a strong Molisch reaction and to contain phosphorus [Euler and Myrbäck, 1930] these properties have been investigated in some of our preparations.

2 cc. of the fraction obtained from the gold precipitate, which contained at least 10 rat day-doses, gave a negative reaction. Of the fractions obtained by the electrodialysis of a purified vitamin B_1 concentrate at p_H 8·5 (Birch and Guha, unpublished results) the fraction obtained in the cathode compartment, which contained at least 40 % of the activity, gave a negative Molisch reaction, while the fraction of the middle compartment gave a positive one. 2 cc. of a preparation obtained from rice polishings, potent for rats in 0·02 cc., kindly supplied by Prof. J. C. Drummond, which had been concentrated up to the platinum stage, still gave a positive reaction. This indicates, incidentally, that certain neutral impurities may be got rid of more readily by electrodialysis under suitable conditions than by metallic fractionation. Unlike co-zymase preparations, however, Drummond's concentrate was practically phosphorus-free. All these observations indicate that vitamin B_1 is probably not very closely related in structure to co-zymase. Also co-zymase is more readily destroyed by heat under certain conditions than vitamin B_1 and is susceptible to the action of nucleotidases. Vitamin B_1 does not appear to be very sensitive to the action of enzymes.

The absorption spectra of certain vitamin concentrates.

It was considered of interest to investigate whether vitamin B_1 concentrates show any selective absorption, which may be attributed to the vitamin. A search of the literature showed that Damianovich [1922] had observed an absorption band between 248 and 260 $\mu\mu$ given by an active brewer's yeast extract, which appeared to indicate pyrimidine compounds. Mukherji [1929] has recently observed that the crystals, which he obtained from rice polishings by Jansen and Donath's procedure, exhibited marked absorption at 390–330 $\mu\mu$.

The absorption spectra of (1) the cathode fraction obtained from yeast by fractionating up to Stage 5 and then electrodialysing at p_H 8·5, and of (2) Drummond's rice-polishings concentrate, were examined with the ultraviolet spectrophotometer of the Hilger pattern (Fig. 6). The yeast concentrate

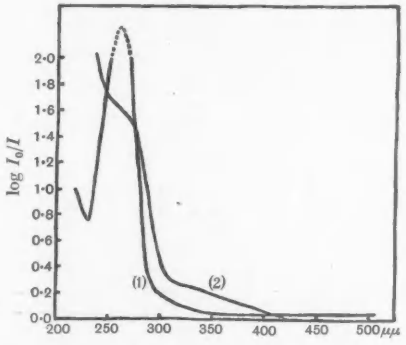

Fig. 6.

Curve (1). The absorption spectrum of the electrodialysed yeast fraction. 100 cc. of the solution contained 40 rat day-doses. Thickness of cell = 2 cm.
Curve (2). The absorption spectrum of Drummond's rice-polishings concentrate. 100 cc. of the solution contained 500 rat day-doses. Thickness of cell = 2 cm.

showed a very strong absorption in the region of 260 $\mu\mu$, which apparently accords with the observation of Damianovich. The same type of band, however, was observed by Dixon and Keilin to be given by a protein precipitate obtained by treatment with sulphur dioxide in course of fractionation for cytochrome from baker's yeast (private communication). This substance was kindly provided by Dr Keilin and, on being tested, it was found to be devoid of vitamin B_1 potency even in 10 mg. doses. This band is therefore not specific for B_1. The nature of the substance causing this absorption is unknown. Drummond's concentrate, which was much more potent than the electrodialysed fraction, did not show this band (it was examined in three dilutions). The band observed by Mukherji was not shown markedly by either of the fractions. It is unlikely that a study of the absorption spectra of vitamin B concentrates will yield any significant results. It should be pointed out that

if vitamin B_1 has the glyoxaline structure as suggested by Jansen and Donath [1926], it may not have any selective absorption, as is indicated by the observations of Marchlewski and Nowotnowna [1925–6] on histidine.

Certain other properties of the vitamin concentrates.

1. *Behaviour towards flavianic acid.*

The fuller's earth extract did not give any appreciable precipitate with flavianic acid even after standing for 5 hours.

2. *Optical activity.*

The phosphotungstic fraction was examined for optical activity with reference to the mercury green line and at 18°, but there was no perceptible rotation, while the fraction obtained at the lead stage showed only a slight activity.

3. *Ninhydrin and Adamkiewicz-Hopkins reactions.*

The preparation obtained after fractionation with phosphotungstic acid and alcohol gave a negative ninhydrin reaction. A fraction purified by electrodialysis at p_H 8·5 gave a very feeble Adamkiewicz-Hopkins reaction, while Drummond's fraction obtained from rice polishings, did not give this reaction.

4. *Pauly reaction.*

The Pauly reaction given by Jansen and Donath's crystals was one of the grounds on which they ascribed a glyoxaline structure to the vitamin [Jansen and Donath, 1926]. This reaction is given rather more strongly by the potent vitamin B_1 concentrates from yeast obtained by Kinnersley and Peters, and both reactions are less intense than that given by histidine [Jansen *et al.*, 1930]. We had observed a strong Pauly reaction with our most potent concentrate from wheat germ [Guha and Drummond, 1929]. The preparations obtained at the platinum stage by the method described in this paper have been found to give a very poor Pauly reaction compared with histidine. This reaction as given by these different concentrates cannot, therefore, be considered as trustworthy evidence about the chemical nature of vitamin B_1.

Discussion.

The fractionation described in this paper gives very regular results, if followed in detail. It is difficult to understand the phenomenon observed at the stage of gold fractionation. The rat-growth tests failed to agree well with the curative tests on pigeons. One explanation might be that there is a difference between the rat-growth factor (or factors) and the pigeon curative factor (or factors). On the other hand, it is necessary to point out that the particular pigeon tests with the gold fractions showed very large individual variations and it is probable that the results are untrustworthy. This error could only be partially eliminated if an unusually large number of pigeons

were used. The results obtained with the rats are much more regular and these indicate that there is an enormous loss of vitamin B_1 at the gold fractionation stage, and the supplementary effect of the fractions obtained from the gold precipitate and filtrate, if any, cannot be expected to be observed under the circumstances. The question therefore must be considered still unsettled.

About the actual chemical nature of vitamin B_1 nothing can yet be said with confidence. Jansen and Donath's crystals have now been stated to contain vitamin B_4 [Jansen *et al.*, 1930]. Williams, Waterman and Gurin [1930] in a recent investigation could not obtain the pure crystals, while Mukherji [1929] states that by following Jansen and Donath's procedure in the main and working with rice polishings he has obtained crystals identical with those described by Jansen and Donath. It is not clear, however, that the crystals were pure. The tests, which were carried out on rice-birds (*Munia maja*) showed that 30 mg. of the crystals per day were effective for protection from "polyneuritis." Lower doses do not appear to have been tried. The Pauly reaction given by different vitamin B_1 concentrates from rice polishings and yeast cannot yet safely be attributed to the vitamin *per se*.

Summary.

1. Adenine, guanine, uracil, *dl*-thyroxine, histamine and adrenaline cannot replace vitamin B_1 in the diet of rats.

2. A fairly standardised method of concentration of vitamin B_1 is described by which, starting from brewer's yeast, a preparation can be obtained at the platinum stage, which promotes good growth in rats in a daily dose of 0·075–0·1 mg. when supplemented by vitamin B_2. Its pigeon-curative day-dose is of the order of 0·047 mg.

3. No clear-cut answer has been obtained to the question whether fractionation by gold chloride would provide any evidence for the splitting of vitamin B_1. The rat-growth tests and pigeon-curative tests of these fractions show serious discrepancies.

4. Vitamin B_1 does not appear to be closely chemically related to cozymase. Its relation to secretin and "bios" is discussed.

5. Some vitamin B_1 concentrates are optically inactive and give negative ninhydrin and Adamkiewicz-Hopkins reactions. The Pauly reaction given by the purer yeast concentrates is comparatively weak. Vitamin B_1 is not apparently precipitated by flavianic acid from a fairly concentrated preparation.

6. The absorption spectra of certain concentrates are described.

I wish to express my thanks to Sir F. G. Hopkins and Prof. J. C. Drummond for their stimulating interest, and also to Dr M. Dixon for his interest and generous help in the work on the absorption spectra of the concentrates.

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CV. INVESTIGATIONS ON VITAMIN B_2.

I. THE SOURCES OF VITAMIN B_2.

II. THE STABILITY OF VITAMIN B_2.

III. THE CHEMISTRY OF VITAMIN B_2.

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Since the recognition of the presence of at least two factors, B_1 and B_2, in the dietary complex known as "vitamin B," little work has been published on the nature and behaviour of vitamin B_2. The present investigation was prompted by the need for fuller knowledge of this subject. Preliminary reports of some of the results have appeared elsewhere [Guha, 1931, 2, 3].

Biological technique.

Young rats of about 50 g. in weight were kept in separate cages with screened bottoms and were fed on the basal diet described in a preceding paper [Guha, 1931, 1]. Each animal received separately in a dish a daily dose of 2 drops of cod-liver oil and of a preparation of vitamin B_1 from the beginning of the experimental feeding period. The B_1-preparation was obtained from brewer's top yeast by extracting with aqueous alcohol and then fractionating with neutral lead acetate [Guha, 1931, 1]. In some experiments the filtrate from the lead precipitate was further purified by adsorption on fuller's earth by the method described before. In others, however, the lead filtrate itself was used as the source of vitamin B_1 and was fed in a daily dose of 0·5 c.c., equivalent to 1–1·5 g. of the fresh yeast. This preparation contained a small amount of vitamin B_2, but as both positive and negative controls were kept, the results were checked throughout. Rats on the above dietary began to decline in weight in 2–4 weeks from the commencement of the experiment, according to the reserve of vitamin B_2 in their bodies. Test fractions were fed at this stage and if growth occurred at a rate of 10–12 g. per week for 2–3 weeks, the fractions were considered to be active. Fig. 1 shows that the B_1-preparation (lead filtrate) cannot by itself produce growth in 0·5 cc., but can produce slow growth in 3–4 times that dose. If, however, 0·5 cc. of the B_1-preparation is supplemented by 1 cc. of a 50 % solution of alkalised marmite [Guha, 1931, 1] as a source of vitamin B_2, good growth is obtained, whereas the alkalised marmite by itself cannot sustain growth even in a daily dose of 4 cc.

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The B_1-preparation made by means of fuller's earth is almost entirely devoid of vitamin B_2 and is probably to be preferred in all tests for vitamin B_2. The lead filtrate, however, is likely to contain other factors [Reader, 1930], which might be an advantage.

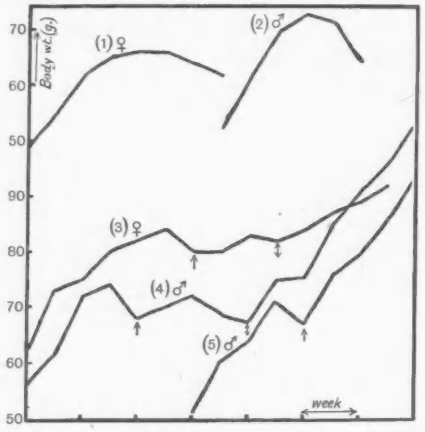

Fig. 1.

Curve (1). 0·5 cc. of vitamin B_1 preparation (lead filtrate) from the beginning of experiment.
Curve (2). 1 cc. of vitamin B_2 preparation (alkalised marmite) from the beginning of experiment.
Curve (3). 0·5 cc. of lead filtrate from the beginning. At ↑ given 1 cc. of lead filtrate. At ↕ given 3·0 cc. of lead filtrate.
Curve (4). 1 cc. of alkalised marmite from the beginning. At ↑ 4 cc. of the same. At ↕ 1 cc. of alkalised marmite *plus* 0·5 cc. of lead filtrate.
Curve (5). 1 cc. of alkalised marmite from the beginning. At ↑ 0·5 c.c. of lead filtrate added.

No case of refection was noticed in these experiments though uncooked rice starch was used in the basal diet. It is our custom, however, to check the results by observing whether the withdrawal of an active supplement is followed by a decline in weight.

I. The sources of vitamin B_2.

It was early realised that the recognition of the dual nature of "vitamin B" required a revision of the "vitamin B" values of foodstuffs, especially where these values were obtained by growth tests on rats. Aykroyd and Roscoe [1929] and Roscoe [1930] have recently assayed vitamin B_2 in certain materials.

The present estimation of vitamin B_2 in certain raw materials was carried out with the purpose of finding out which of these would be most useful for the extraction of the vitamin. Aqueous extracts of brewer's top yeast, baker's yeast, ox-liver, beef-muscle, commercial liver extract (Eli Lilly, No. 343)[1], and milk powder were examined.

[1] I am indebted to Dr C. Elvehjem for the commercial liver extract used in this research.

Brewer's yeast, baker's yeast, ox-liver and beef-muscle were extracted under identical conditions, the liver and muscle being finely minced before extraction. 300 g. of each of them were stirred gradually into 480 cc. of boiling distilled water and the boiling continued for 3 minutes.

These were filtered under suction and washed each with 100 cc. water. The muscle gave a practically colourless solution, while the liver yielded an opalescent brownish liquid with a faint green fluorescence.

Eli Lilly's commercial liver extract was fed both in the solid state and also as an aqueous extract which was prepared by stirring 50 g. of the liver extract with 200 cc. of cold water. This was filtered under suction from a small amount of insoluble residue which was washed with a little water. The deep brown-red filtrate was made up to 250 cc. to make a 20 % solution. The residue was apparently a remnant of the liver protein. It dissolved in $N/10$ NaOH and was precipitated by the careful addition of $N/10$ HCl at p_H about 4·6, which is close to the isoelectric point of the liver protein. Both the residue and the aqueous extract were tested for activity.

"Glaxo" milk powder, which was prepared from English winter milk, was fed both in the solid state and in an aqueous medium.

The results of these tests are shown in Figs. 2 and 3. It will be observed

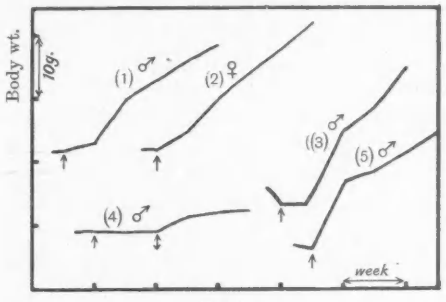

Fig. 2.

Curve (1). ↑ dose≡1·4 g. fresh brewer's yeast.
Curve (2). ↑ dose≡0·7 g. fresh baker's yeast.
Curve (3). ↑ dose≡0·94 g. fresh baker's yeast.
Curve (4). ↑ dose≡0·9 g. fresh beef muscle; ↕ dose≡1·8 g. fresh beef muscle.
Curve (5). ↑ dose = 0·4 g. milk powder.

that the extract of baker's yeast is markedly richer in vitamin B_2 than that of the brewer's yeast, the former giving as good growth in a dose corresponding to 0·7–0·9 g. of fresh yeast as the latter in a dose equivalent to 1·4 g. Some hope was entertained about milk powder as a raw material for the fractionation of vitamin B_2, as the major constituents of this material are known. But it proved to be active only in a dose as large as 0·4 g. The muscle extract was comparatively inactive, which is rather surprising, considering that, according to Goldberger, lean meat is a rich source of the antipellagra factor. Vitamin B_2 may, however, be present in muscle in a combination from which

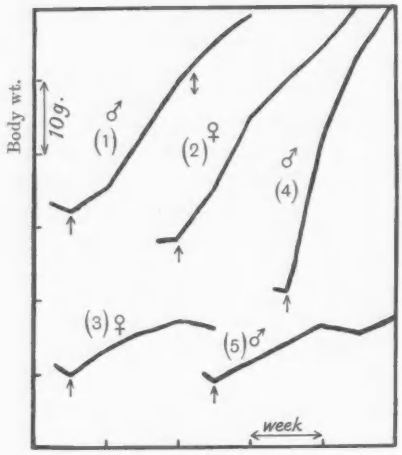

Fig. 3.

Curve (1). Aqueous extract of fresh ox-liver; ↑ dose≡0·3 g. of fresh liver (containing 6·0 mg. organic material); ↕ dose≡0·15 g. of fresh liver.
Curve (2). A cold aqueous extract of Eli Lilly's commercial liver concentrate; ↑ dose≡50 mg. of the liver concentrate.
Curve (3). The insoluble residue obtained after a cold aqueous extraction of the commercial liver concentrate; ↑ dose≡0·25 g. of the concentrate.
Curve (4). Commercial liver concentrate; ↑ dose=0·15 g.
Curve (5). Shows the vitamin B_1 potency of the cold aqueous extract of commercial liver concentrate; ↑ dose≡0·12 g. of the concentrate.

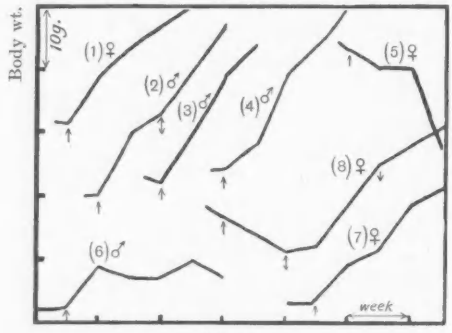

Fig. 4.

Curve (1). Aqueous extract of commercial liver concentrate, autoclaved at p_H 9·0 for ½ hour; ↑ dose≡60 mg. of the concentrate.
Curve (2). Same extract, autoclaved at p_H 9·0 for 3 hours; ↑ dose≡90 mg. of the concentrate; ↕ dose≡60 mg. of the concentrate.
Curve (3). Same extract, autoclaved at p_H 7·4 for 3 hours; ↑ dose≡60 mg. of the concentrate.
Curve (4). Same extract, autoclaved at p_H 6·2 for 3 hours; ↑ dose≡60 mg. of the concentrate.
Curve (5). Vitamin B_1 potency of the same extract, autoclaved at p_H 9·0 for ½ hour; ↑ dose≡180 mg. of the concentrate.
Curve (6). Brewer's yeast extract, autoclaved at p_H 9·0 for 3 hours; ↑ dose≡4·6 g. of fresh yeast.
Curve (7). Same extract, autoclaved at p_H 6·25 for 3 hours; ↑ dose≡1·7 g. of fresh yeast.
Curve (8). Aqueous extract of fresh ox-liver, autoclaved at p_H 9 for 3 hours; ↑ dose≡0·3 g. of fresh liver; ↕ dose≡0·9 g. of liver; ↓ dose≡0·6 g. of liver.

it is difficult to separate it by boiling water. The muscle residue was not tested for its activity. The aqueous extract of fresh ox-liver was extremely potent, being active in a daily dose containing less than 6 mg. total solids, which were equivalent to about 0·3 g. of the fresh liver. This extract should serve as a promising source of vitamin B_2 for fractionation and other purposes.

The cold aqueous extract of the commercial liver concentrate was highly active in a daily dose of 40–60 mg. The residue was practically inactive. This 20 % aqueous extract was used for the chemical study described in this paper. This material has some advantages over yeast, which has been so far generally used as a source of vitamin B_2. It is more potent, and it can be readily prepared from a stock of the solid liver concentrate whenever required. This ensures a greater uniformity of results. It is, moreover, comparatively poor in vitamin B_1, which is an advantage for certain purposes. The 20 % aqueous extract keeps well in the refrigerator.

II. The stability of vitamin B_2.

There has lately been a serious disagreement among different workers about the stability of vitamin B_2, especially to heat in an alkaline medium. Goldberger's work had shown that the factor concerned in the prevention of pellagra was relatively thermostable [Goldberger *et al.*, 1926]. In fact, the relative thermostability of vitamin B_2 has so far been the main factor in its differentiation from the more heat labile vitamin B_1. Other workers have also found that the vitamin B_2 potency of the commercial yeast extract, marmite, is not very seriously reduced by autoclaving for 3 hours at 120° at an alkaline reaction [Hassan and Drummond, 1927; Guha and Drummond, 1929; Narayanan and Drummond, 1930]. That a preparation of alkaline-autoclaved marmite can supplement a concentrated preparation of vitamin B_1 for the growth of vitamin B-deficient rats has been confirmed by us in hundreds of cases. On the other hand, other workers have stated that autoclaving in an alkaline medium destroys vitamin B_2 in brewer's yeast and its extracts, made by boiling with 0·01 % acetic acid, almost entirely [Williams, Waterman and Gurin, 1929; Chick and Roscoe, 1930].

It appeared to the writer at the outset that these discrepancies about the stability of vitamin B_2 might be due to the different behaviour of vitamin B_2 when present in association with different substances. That such is the case with vitamin B_1 is now well established [Kinnersley and Peters, 1928; Guha and Drummond, 1929]. An investigation was, therefore, instituted to find out the vitamin B_2 potency of some of the extracts, described in Part I, after autoclaving under identical conditions.

Aqueous extracts of brewer's top yeast, fresh ox-liver and the commercial liver concentrate were made as described and their p_H adjusted with $N/2$ NaOH. The treatment undergone by the extracts and the results on testing are summarised in the following table (see also Fig. 4).

Nature of material	Period of autoclaving (hours)	Temp. of autoclaving	Initial p_H	Final p_H	Percentage of inactivation (roughly)
Extract of fresh ox-liver	3	124–125°	9·0	6·2	75
Extract of commercial liver concentrate	3	124–125°	9·0	6·1	0
,, ,,	$\frac{1}{4}$	124–125°	9·0	6·5	0
,, ,,	3	124–125°	7·4	4·87	0
,, ,,	3	124–125°	6·2	4·65	0
Brewer's yeast extract	3	124–125°	9·0	7·5	90
,, ...	3	124–125°	6·25	5·62	0

Thus the curious fact is revealed that while vitamin B_2 in commercial liver extract and also in marmite [Hassan and Drummond, 1927] possesses a very high degree of stability, vitamin B_2 in the extracts made by boiling liver and fresh yeast with water is very markedly unstable to alkaline autoclaving. As the 20 % solution of the commercial liver extract contained about 5 times more solids than the brewer's yeast extract, the possibility could not be excluded that the greater dilution of the latter was helping its inactivation. The solution of the liver concentrate was therefore diluted 5 times and autoclaved at p_H 9 for 3 hours at 124–125°, when the p_H changed to 7·6. There was very slight inactivation, if any at all. It is probable that the stability of vitamin B_2 in a given preparation is due to the presence of some kind of protective material in it. Two possibilities present themselves: (1) that the commercial liver extract and marmite contain something which is added to them by the manufacturers and which has a protective action on vitamin B_2, (2) that there is a natural protective agent present in fresh liver and yeast, which is removed by the method of boiling with water, but which is not removed entirely in the manufacturing methods employed. The writer is inclined to think that the latter is the more probable explanation. The protective action of certain colloids, for instance of gelatin and saponin on gold sol, is well known.

Half an hour's autoclaving of the 20 % aqueous solution of the commercial liver extract at p_H 9 at 124–125°, while it did not affect vitamin B_2, destroyed vitamin B_1 almost entirely. Such a preparation would, therefore, appear very suitable for purposes where vitamin B_2, free from vitamin B_1, is needed. As this preparation contains much less solid matter and is apparently more palatable to the rats, it is to be preferred to autoclaved marmite.

III. The chemistry of vitamin B_2.

Few facts have been recorded in the literature on the concentration and chemistry of vitamin B_2. Chick and Roscoe [1929] observed that it was precipitated to a large extent from an aqueous yeast extract by neutral lead acetate. Narayanan and Drummond [1930] obtained a concentrate from yeast by successive fractionations with lead acetate and alcohol. Levene [1930] reports that he has obtained a preparation of vitamin B_2, which is potent for the growth of young rats in a daily dose of 0·7 mg.

The 20 % aqueous solution of Eli Lilly's liver extract, described in Part I, or the solid extract itself was used as the material for the present study. The potency of a fraction was, as usual, established by testing on at least two animals, frequently more. In no case was a serious discrepancy observed between the responses of two rats to a given fraction.

1. *Treatment with alcohol.* On treatment of 5 g. of the solid liver extract with 50 cc. of 58 % alcohol, most of the material dissolved giving a red solution. The residue (0·37 g.) was dissolved in water with a few drops of *N*/10 NaOH. About 65 % of the activity passed into the alcoholic extract, the rest being left in the residue (Fig. 5). There was no marked loss of activity

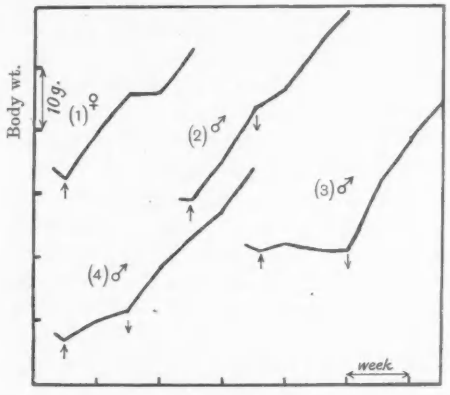

Fig. 5.

Curve (1). Fraction insoluble in 58 % alcohol; ↑ dose≡0·125 g. of the liver concentrate.
Curve (2). Fraction soluble in 58 % alcohol; ↑ dose≡0·125 g. of the liver concentrate; ↓ dose≡0·100 g. of the concentrate.
Curve (3). ↑ Picric acid precipitate, dose≡0·2 g. of the liver concentrate; ↓ picric acid filtrate, dose≡50 mg. of the liver concentrate.
Curve (4). Acetone-precipitated fraction; ↑ dose≡75 mg. of the liver concentrate; ↓ dose≡0·125 g. of concentrate (containing about 8 mg. organic material).

[*cf.* Chick and Copping, 1930]. But the treatment with 58 % alcohol does not apparently cause a clear-cut separation of vitamin B_2 from inactive material.

2. *Treatment with picric acid.* 100 cc. of the 20 % aqueous solution of the liver concentrate were treated with a moderate excess of a hot saturated aqueous solution of picric acid. After cooling, the clear red supernatant fluid was removed from the somewhat gummy precipitate, both were decomposed by hydrochloric acid and the picric acid was repeatedly extracted with ether and then with benzene. The material obtained from the picric acid precipitate was entirely inactive, while that from the picric acid filtrate retained practically all the activity (Fig. 5). This experiment shows that the vitamin is almost insoluble in ether and benzene. Most of the vitamin B_1 potency of the original liver extract was also obtained in the picric acid filtrate.

Fractionation with acetone. The picric acid filtrate which had a p_H of about 1, was treated with 6 times its volume of acetone, when an oily precipitate came down. After leaving the mixture in the refrigerator for 2 days in a corked flask, the clear reddish-orange supernatant liquid was poured off from the gummy brown residue. The residue, after being dried with a stream of air, was found to contain a little more than 50 % of the activity (Fig. 5). The mother-liquor was brought to p_H 6 and again treated with an excess of acetone, when a second oily precipitate separated, but both this precipitate and the filtrate were inactive.

The active fraction obtained by the first precipitation with acetone gave a negative purine test, a doubtful Millon's test and positive biuret and Adamkiewicz-Hopkins reactions. It also gave a very marked Molisch reaction, a fairly positive reaction with Tollen's reagent and a negative one with Bial's. It reduced Benedict's reagent and gave a positive sulphur reaction on prolonged boiling with lead acetate and concentrated sodium hydroxide.

The active picric acid filtrate fraction does not give a precipitate with flavianic acid even after standing for 3–4 hours.

3. *Action of benzoyl chloride.* 30 cc. of the 20 % solution of the liver concentrate were shaken up with 4 cc. of benzoyl chloride and excess of 20 % NaOH in the usual way. A considerable quantity of a yellow precipitate

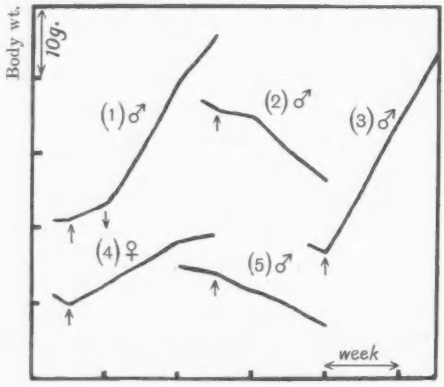

Fig. 6.

Curve (1). Filtrate from the precipitate obtained with benzoyl chloride; ↑ dose≡90 mg. of the liver concentrate; ↓ dose≡180 mg. of the concentrate.
Curve (2). The precipitate formed with benzoyl chloride; ↑ dose≡0·6 g. of the concentrate.
Curve (3). Filtrate from the precipitate obtained with nitrous acid; ↑ dose≡60 mg. of concentrate.
Curve (4). Filtrate from the precipitate obtained with phosphotungstic acid; ↑ dose≡0·3 g. of the concentrate.
Curve (5). Phosphotungstic acid precipitate fraction; ↑ dose≡0·3 g. of the concentrate.

came down, which was removed and dried in a desiccator (weight = 3·5 g.). The filtrate was treated with hydrochloric acid and the benzoic acid removed with ether. This filtrate retained about one-third of the activity, while the precipitate was inactive (Fig. 6).

4. *Action of nitrous acid.* Contrary to Levene [1928], Chick [1929] and Narayanan and Drummond [1930] reported that vitamin B_2 of yeast extracts was stable to nitrous acid. Vitamin B_2 of the 20 % solution of the liver concentrate was also found to be neither precipitated nor inactivated by nitrous acid.

30 cc. of the 20 % solution, acidified with hydrochloric acid, were treated in the usual way with a sufficient volume of a concentrated solution of sodium nitrite, as indicated by starch iodide paper. This was warmed at 60° for half an hour and the nitrous fumes expelled by boiling for 2 minutes in an open beaker. The precipitate, which had come down by treatment with nitrous acid, was inactive, while the filtrate retained the entire activity (Fig. 6).

5. *Reaction with phosphotungstic acid.* 25 cc. of the solution of liver extract were made 5 % acid with concentrated H_2SO_4 and treated with 45 cc. of saturated phosphotungstic acid in 5 % H_2SO_4. After standing overnight, the precipitate, which had removed most of the pigment from the solution, was filtered off. Both the precipitate and filtrate were freed from phosphotungstic acid by means of baryta, barium being finally removed with sulphuric acid. The precipitate fraction was entirely inactive, while the material obtained from the phosphotungstic filtrate was only very slightly active (Fig. 6). A combination of the precipitate and filtrate fractions did not give any more satisfactory results. It is highly probable that the vitamin was removed by adsorption on the surface of barium phosphotungstate and barium sulphate.

6. *Treatment with baryta.* 50 cc. of the 20 % solution were treated with 60 cc. of saturated baryta and left overnight. The resulting precipitate was removed and freed from barium by sulphuric acid (fraction *a*). The filtrate and washings from the barium precipitate were treated with an equal volume of alcohol and allowed to stand for 3 hours. The precipitate which had separated was filtered off and freed similarly from barium (fraction *b*). The filtrate from the second barium precipitate, which was still highly pigmented, was acidified with sulphuric acid and concentrated under reduced pressure (fraction *c*). Fractions (*a*) and (*b*) were inactive, while fraction (*c*) retained a little more than 30 % of the original activity (Fig. 7).

7. *Reaction with lead acetate.* 50 cc. of the aqueous liver extract, which was normally at p_H 4·6, on treatment with 20 cc. saturated neutral lead acetate (a slight excess) deposited a small quantity of precipitate. After standing overnight, this was removed and both precipitate and filtrate were decomposed by means of sulphuric acid. Vitamin B_2 was found to be partially precipitated by this procedure (Fig. 7).

Since treatment with lead acetate at p_H 4·6 did not cause a complete separation of vitamin B_2, the experiment was repeated at p_H 7 with no better results. The lead precipitates in either case were practically free from vitamin B_1. The lead precipitate fraction can thus be used as a source of vitamin B_2, free from vitamin B_1, but the method does not appear to have any great advantage over that described in Part II.

The lead precipitate fraction obtained at p_H 4·6 may be further fractionated by bringing it to p_H 6·8 and then treating it with 6 volumes of acetone. The active material is again precipitated as in the case of the picric acid precipitate.

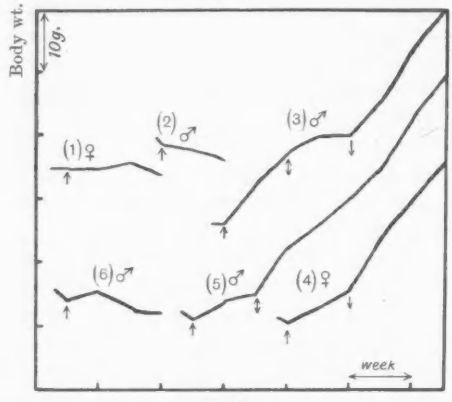

Fig. 7.

Curve (1). Fractionation with baryta; fraction *a*; ↑ dose≡0·2 g. of liver concentrate.
Curve (2). Fractionation with baryta; fraction *b*; ↑ dose≡0·2 g. of liver concentrate.
Curve (3). Fractionation with baryta; fraction *c*; ↑ dose≡0·2 g. of concentrate; ↕ dose≡0·1 g. of concentrate; ↓ dose≡0·15 g. of concentrate.
Curve (4). Lead filtrate; ↑ dose≡0·1 g. of concentrate; ↓ dose≡0·2 g. of concentrate.
Curve (5). Lead precipitate; ↑ dose≡0·1 g. of concentrate; ↕ dose≡0·15 g. of concentrate.
Curve (6). Shows the vitamin B_1 potency of the lead precipitate fraction; ↑ dose≡0·2 g. of concentrate.

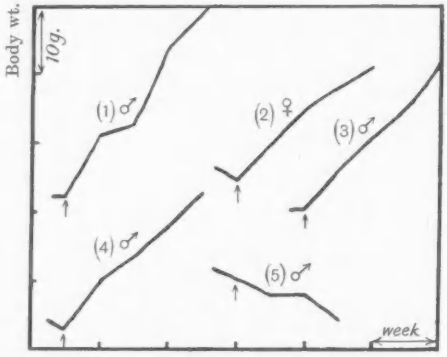

Fig. 8.

Curve (1). Silver nitrate precipitate fraction; ↑ dose≡0·1 g. of liver concentrate.
Curve (2). Silver nitrate filtrate fraction; ↑ dose≡0·3 g. of liver concentrate.
Curve (3). Filtrate from precipitate with litharge; ↑ dose≡75 mg. of concentrate.
Curve (4). Non-esterified fraction; ↑ dose≡0·15 g. of concentrate.
Curve (5). Esterified fraction; ↑ dose≡0·4 g. of concentrate.

8. *Reaction with silver nitrate.* 20 cc. of the aqueous liver extract were treated with 13 cc. of a 25 % solution of silver nitrate, the mixture left over-

night in the dark and the precipitate filtered off. Silver was removed from both the precipitate and filtrate by treatment with HCl and subsequent centrifugation (Fig. 8). The precipitate fraction was much more potent than the filtrate fraction. It appears that this method causes a more effective separation of vitamin B_2 from inactive material than does the reaction with lead acetate.

A portion of the silver precipitate fraction was brought to p_H 9 and autoclaved for 3 hours at 124–125°. The p_H changed to 8·0 and the preparation was found to be at least half inactivated. This shows that purification by certain methods may make the vitamin less stable to alkaline autoclaving, probably by removing some protective material (*cf.* Part II).

9. *Treatment with litharge.* 25 cc. of the aqueous liver extract were shaken up with 3 g. (excess) of litharge. After filtration the residue and the filtrate were freed from lead by sulphuric acid. Practically all the activity was present in the filtrate (Fig. 8).

10. *Esterification with ethyl alcohol.* 4 g. of the solid liver extract were covered with 120 cc. absolute alcohol and treated with dry hydrogen chloride in the usual way for 5 hours. In the last hour the temperature of the reaction vessel was maintained at 50–60°. Nearly all the material dissolved giving a dark brown-red solution. The contents of the flask were poured into a beaker and chilled to 0°. Two volumes of water were slowly added and then silver carbonate and silver oxide. When the p_H of the solution was still below 1, the precipitate, which appeared to consist wholly of silver chloride, was filtered off under suction and well washed and the washings added to the filtrate. The dark red solution was again brought to 0° and then very slowly treated with 20 % sodium hydroxide with constant stirring until the p_H was about 9·5 (the temperature should not rise above 5°). The solution which now measured 525 cc., was immediately extracted 4 times with a total volume of about 750 cc. ether. The ethereal layer was pale yellow, most of the pigment being in the aqueous layer. The ethereal layer was washed with very dilute hydrochloric acid and then with water, which removed some more of the colouring matter. After evaporating the ether by means of an electric fan at 30° the residue was taken up in water (fraction *a*). The aqueous layer was acidified with HCl, concentrated under reduced pressure, and filtered from the precipitated sodium chloride. As it was, however, still extremely saline and could not be fed to the animals, it was saturated with hydrogen chloride at 0°. After removing the large amount of precipitated sodium chloride the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in water, brought to p_H about 6 and fed to the animals (fraction *b*). Fraction *a* was inactive, while about 30–40 % of the original activity was present in fraction *b* (Fig. 8). The recovery appears to be good enough to warrant the conclusion that the vitamin is not esterifiable under the aforesaid conditions of experiment.

11. *Adsorption on charcoal.* It has been the experience of other workers

[Salmon, Guerrant and Hays, 1928; Narayanan and Drummond, 1930] that vitamin B_2 of yeast extracts is tenaciously adsorbed by certain substances, from which it is difficult to remove by elution. Such has also been the experience of the writer in using charcoal for the adsorption of vitamin B_2 from the liver concentrate.

100 cc. of the aqueous liver extract (p_H *circa* 4·6), were shaken up with 6·7 g. of norite charcoal for 5–7 minutes. After filtration, the charcoal weighed 12–15 g., and was potent in doses corresponding to 80–100 mg. of the original liver extract. The filtrate was practically inactive (Fig. 9).

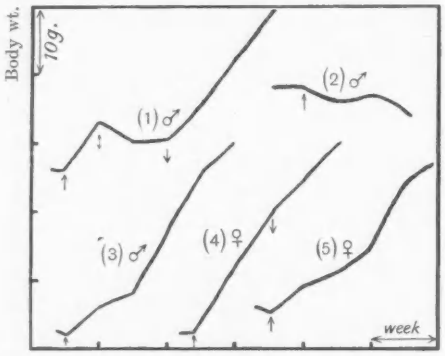

Fig. 9.

Curve (1). Activated charcoal; ↑ dose≡0·1 g. of liver concentrate; ↕ dose≡75 mg. of concentrate; ↓ dose≡90 mg. of concentrate.
Curve (2). Charcoal filtrate; ↑ dose≡0·18 g. of concentrate.
Curve (3). SO_2-treated preparation; ↑ dose≡60 mg. of the concentrate.
Curve (4). O_3-treated preparation; ↑ dose≡80 mg. of concentrate; ↓ dose≡60 mg. of concentrate.
Curve (5). H_2O_2-treated preparation; ↑ dose≡80 mg. of concentrate.

5 g. portions of this activated charcoal were extracted with 50 cc. each of 0·1 *N* HCl, 0·1 *N* NaOH, 30 % ethyl alcohol, containing 0·5 cc. concentrated hydrochloric acid and 45 % alcohol, containing 0·5 cc. of 20 % NaOH. The extractions were carried out by heating under reflux on a water-bath for 1 hour. The extract in every case was practically inactive. The charcoal residues were in some cases active, but the activity had been materially reduced. 5 g. of the activated charcoal were also extracted successively with 200 cc., 200 cc. and 100 cc. of 30 % propyl alcohol, the flask being heated each time at 75 ° for 10 minutes. The extract was slightly active, and the residue practically inactive. Another portion of 5 g. of the activated charcoal was extracted twice with 125 cc. of 0·2 % saponin ("pure," B.D.H.) in the cold, and the mixture centrifuged, but the centrifugate was inactive.

12. *Stability to sulphur dioxide.* Sulphur dioxide was passed into 20 cc. of the aqueous liver extract for 4 hours. The excess of SO_2 was boiled off in an open beaker. The vitamin is completely stable to this treatment (Fig. 9).

13. *Stability to hydrogen peroxide.* 20 cc. of the aqueous extract were treated with 5 cc. of Merck's perhydrol, heated for 1½ hours at 70° and then evaporated to dryness in an open basin in a water-bath. The residue was dissolved in water. No very marked inactivation occurred (Fig. 9).

14. *Stability to ozone.* 20 cc. of the aqueous extract were treated with a rapid stream of ozonised oxygen for 70 minutes. The colour of the solution gradually faded to a lemon-yellow. This was concentrated under reduced pressure. The vitamin was fairly stable to such treatment (Fig. 9).

15. *Action of trypsin.* 80 cc. of the aqueous liver extract were brought to p_H 8. 1 g. powdered trypsin (B.D.H.), which had been found in this laboratory to be active, was added. The mixture was incubated after the addition of a little toluene for 7 days at 37°. Another 80 cc. of the aqueous liver extract was similarly incubated without trypsin as a control. The p_H of the digested solution had changed to 7·2 in 7 days. Both the solutions were acidified with hydrochloric acid, concentrated *in vacuo* to remove toluene and tested. Both of them were active. The trypsin powder itself exhibited no activity even in relatively large doses.

16. *The behaviour of vitamin B_2 on electrodialysis.* Preliminary experiments carried out with Mr T. W. Birch on the electrodialysis of vitamin B_2 of the aqueous liver extract, indicate that the vitamin is probably a neutral substance. It remains concentrated in the middle compartment under the conditions tried, while small quantities appear to be present in both the anode and cathode compartments, this being apparently due to diffusion through the parchment membrane.

Discussion.

The behaviour of vitamin B_2 of the liver extract to basic precipitants like phosphotungstic acid, benzoyl chloride, picric acid, *etc.* indicates that the vitamin is probably not a basic substance. On the other hand, the fact that esterification of the extract leaves a fair amount of the vitamin in the non-esterified fraction, while the esterified fraction is inactive, argues against the acid nature of the vitamin. The occurrence of both vitamin B_2 and the factor specific for pernicious anaemia in large quantities in liver raises the question of a relation between the two substances. The fact that a lowering of the erythrocyte count has been observed in vitamin B_2 deficiency [Sure, Kik and Smith, 1931] also lends point to this question. The general chemical behaviour of the vitamin, as shown in this paper, is however in contrast to that of the factor for pernicious anaemia, which has recently been stated by Dakin, West and Howe [1930] to be a compound of β-hydroxyglutamic acid and l-γ-hydroxyproline.

The idea gained from general chemical evidence that vitamin B_2 is probably a neutral substance is apparently corroborated by the experiments on the electrodialysis of the vitamin. The precipitation of a large portion of the

activity with lead acetate and silver nitrate is probably due to the adsorption of the vitamin by the precipitates formed.

Certain other observations.

Among the rats, which were kept for periods of 10 weeks or so on a vitamin B_2-deficient diet in course of the experiments described in this paper, with only occasional administrations of small doses of vitamin B_2, a considerable proportion developed a curious form of depilation. The fur fell off not in patches but uniformly in such a manner as to give the rats a "close-cropped" appearance [Guha, 1931, 2]. Only in a very small proportion of cases did lesions appear near the mouth and on the paws. The hair began to grow within 2 or 3 days of the administration of the liver concentrate as a source of vitamin B_2 and within 7 or 8 days assumed the normal "lying-down" appearance. This condition of depilation has occurred fairly regularly when the deprivation of vitamin B_2 is not complete but sufficiently severe to cause the weight of the animals to remain between 60 and 80 g. over a period of 10–12 weeks [*cf.* Leader, 1930; Sure and Smith, 1931].

The above symptoms did not improve nor did growth resume on feeding lactalbumin, haemoglobin or haemin[1] in daily doses of 1 g., 0·5 g. and 25 mg. respectively [*cf.* Kollath, 1929; Bliss, 1930; Bliss and Thomason, 1931].

Summary.

1. The values of the following as sources of vitamin B_2 have been investigated—milk powder and aqueous extracts of brewer's yeast, baker's yeast, fresh ox-liver, beef-muscle and Eli Lilly's liver concentrate No. 343. The fresh ox-liver extract is apparently the most potent of these. An aqueous extract of the liver concentrate is also very potent, being effective in a daily dose of 40–60 mg. for the growth of young rats. The advantages of this liver concentrate over yeast as a source of vitamin B_2 are pointed out.

2. The stability of vitamin B_2 preparations obtained from different sources towards heat and alkali shows curious discrepancies. Thus the vitamin B_2 in aqueous extracts of yeast and fresh ox-liver is much less stable than that in marmite and in the commercial liver concentrate. It is probable that the stability of certain preparations is connected with the presence of some kind of protective material in them. An aqueous extract of the liver concentrate autoclaved at p_H 9 for half an hour at 124–125° provides an excellent source of vitamin B_2 free from vitamin B_1.

3. A chemical study of vitamin B_2 in a cold aqueous extract of the liver concentrate has been made. Picric acid, benzoyl chloride, phosphotungstic acid and flavianic acid do not precipitate the vitamin. Nitrous acid neither precipitates nor inactivates it. Lead acetate and silver nitrate precipitate it partially. Esterification leaves about 40 % of the vitamin in the non-esterified

[1] I am indebted to Mr Meldrum for this preparation of haemin.

fraction, the esterified fraction being inactive. It is not attacked by trypsin. Norite charcoal adsorbs the vitamin at the normal p_H (4·6) of the aqueous extract of the liver concentrate. It was not possible to elute it effectively by aqueous alcohol, 30 % propyl alcohol or by dilute saponin. On the basis of the present evidence it may be provisionally concluded that the vitamin is probably not a base, acid or peptide, but a neutral substance. Preliminary experiments on the electrodialysis of the vitamin support this tentative conclusion. The partial precipitation by lead acetate and silver nitrate is probably due to the adsorption of the vitamin on the precipitates formed. The vitamin is stable to sulphur dioxide, hydrogen peroxide and ozone.

4. Some of the rats which were maintained at a weight between 60 and 80 g. over periods of 12 weeks or so, and were not undergoing a drastic deprivation of vitamin B_2, developed a curious form of depilation, which was cured by administration of the liver extract.

5. Haemin, haemoglobin and lactalbumin could not ameliorate the above symptoms or produce growth in absence of vitamin B_2.

I desire to express my gratitude to Sir F. G. Hopkins for his interest in this work and to thank Mr A. Ward and Miss Ruby Leader for their skilful assistance with the animals.

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CVI. OBSERVATIONS ON CERTAIN FACTORS NECESSARY FOR THE NORMAL NUTRITION OF THE RAT.

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Introduction.

In course of attempts made to isolate vitamin B_1 [Guha and Drummond, 1929], it was observed early in 1928 that if in these experiments the animals were kept in a state of more or less stunted growth by alternate administration and withdrawal of vitamin B_1 concentrates over a period of 10–12 weeks, the subsequent prolonged administration of vitamin B_1 concentrates of known potency (as determined by pigeon curative tests and growth tests on younger rats) did not permit continued good growth in these animals. Growth was not effectively restored by increasing the doses of vitamin B_1 and B_2 concentrates (the latter being supplied as alkaline-autoclaved marmite). But if they were replaced by relatively small quantities of whole brewer's yeast, dried at 35°, or of marmite, the animals responded with much more continued growth [Guha and Drummond, 1929, p. 896, footnote]. Even so, growth ceased after some time at a point well below the normal. At this stage, milk was tried, partly empirically and partly with the classical experiments of Hopkins [1912] in mind, with distinctly beneficial results. These preliminary observations suggested the possibility that in yeast and in milk we might be dealing with factors other than the known vitamins. As, however, other explanations might well be given for the observed results, these preliminary experiments were followed up and an investigation carried out during the last 3 years with reference to the following points.

(1) Can the observed effect be ascribed to a better protein in the supplement or to increased amounts of the known vitamins A, B_1, B_2, C, D or E?

(2) Is the increased growth associated with increased food intake? If so, would it be possible to have increased growth with restricted food intake?

(3) To extract, if possible, the new factor (or factors) from natural sources and define its chemical character.

In this investigation over one hundred rats were used, some of which were kept under observation for experimental periods exceeding 35 weeks. Extensive food-intake records were kept. No attempt was made to find if the

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absence of the new factor (or factors) would affect breeding and lactation. It should be emphasised that entirely regular results were not obtained, but the effect of the factors concerned was well outside the limits of individual biological variations and was sufficiently regular to warrant the conclusions reached. It may also be pointed out that part of this work was carried out at University College, London, and part at the Biochemical Laboratory, Cambridge, with animals reared on different diets. The maximum attainable weights of the stock rats also differ. Still, the results obtained at the two laboratories agree fairly, and the effects of the absence or administration of the new factors are similar. Unfortunately, not many experiments could be carried out on the extraction of the factors concerned and no suitable method has yet been devised for this purpose. A preliminary report of this work has appeared elsewhere [Guha, 1930].

Since 1926 a number of papers have appeared bearing more or less directly on the same subject [Osborne and Mendel, 1926; Palmer and Kennedy, 1927; Evans and Burr, 1927, 1; Hunt, 1928; Reader, 1928, 1929, 1930; Coward, Key and Morgan, 1929; Mason, 1929; Chick and Copping, 1930]. This work is discussed later in relation to the present investigation.

Experimental.

The rats were kept in separate cages with screened bottoms, and supplements were given, wherever necessary, in separate dishes and, in general, the procedure described by Guha and Drummond [1929] was followed. The rats used at University College, London, were raised on a diet of the following composition:

	%
Maize meal	63
Whole wheat	20
"Casein" (B.D.H. "light white") ...	9
Dried yeast powder	4
Calcium carbonate	3
Common salt	1

Some sheep-liver was supplied once a week. These rats were normally capable of attaining a maximum weight of 300 g. for males and about 210 g. for females, which usually took about 30–34 weeks. The rats at the Cambridge laboratory were bred on a dietary of brown bread, fresh milk and whole wheat, and usually reached a maximum weight of 350–360 g. for males and about 250 g. for females. The animals used at University College and those at Cambridge are designated as "A Class" and "B Class" rats respectively. They were all of the black and white strain. In the following experiments 2 drops of cod-liver oil were given daily to each rat in a dish, unless otherwise mentioned.

A. *Experiments showing the effect of vitamin B_1 and B_2 preparations, yeast, marmite and milk, on rats, whose growth had been artificially stunted over a considerable period.*

Fig. 1 illustrates cases of A Class rats, in which, owing to the alternate administration and withdrawal of vitamin B_1 preparations, the rats were kept at a subnormal weight over considerable periods. These rats were throughout on a basal diet composed of 75 % rice starch, 21 % "light white casein"

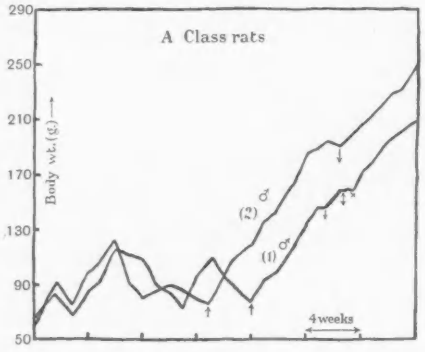

Fig. 1.

Curve (1). ↑ 0·5 cc. of a vitamin B_1 concentrate from wheat germ *plus* 1 cc. of 50 % alkalised marmite; ↓ the dose of each doubled; ↕ the dose of each trebled; × 0·5 g. dried brewer's yeast daily.

Curve (2). ↑ 0·5 g. fresh marmite daily; ↓ 15 cc. of milk in addition.

(B.D.H.) and 4 % salt mixture (McCollum). In addition, they received daily doses of 2 drops of cod-liver oil of proved potency and 1·0 cc. of a 50 % solution of alkaline-autoclaved marmite [Guha and Drummond, 1929]. After the expiration of 10–12 weeks from the beginning of experimental feeding, the administration of a vitamin B_1 concentrate, in the form of a fuller's earth extract obtained from wheat germ [Guha and Drummond, 1929] or of a charcoal concentrate obtained from brewer's yeast by the acid-alcohol method of Kinnersley and Peters [1928], could not maintain continued growth. Doubling or trebling the dose of the vitamin B_1 concentrate and of the alkalised marmite produced only a temporary response. The increased effect of brewer's yeast (dried in the laboratory at 35° by means of an electric fan), and of fresh untreated marmite over vitamin B_1 and B_2 preparations, and of fresh pasteurised milk over marmite was quite noticeable. That the vitamin B_1 concentrates used in these experiments were highly potent in small doses (0·5 cc.) was shown by growth tests on younger rats. The activity of the fuller's earth extract was also checked by curative tests on pigeons, the day-dose being found to be of the order of 0·1–0·15 cc. That autoclaved marmite affords a very potent preparation of vitamin B_2 has also been demonstrated

[Hassan and Drummond, 1927] and has been confirmed by the writer on numerous occasions [Guha, 1931].

B. *Experiments demonstrating the increased effect of dried brewer's yeast over that produced by relatively large doses of vitamin B_1 and B_2 preparations on normal growing rats.*

While the effects of yeast and milk, described in Section A, were suggestive and were actually first observed as a side-issue in our work on the fractionation of vitamin B_1, it was obvious that the fact that these observations were made under conditions in which growth was artificially inhibited over a fairly long period would complicate the conclusions. Experiments were, therefore, carried out with young growing A Class rats, which were given vitamin B_1 and B_2 preparations of known potency from the start of the experimental feeding period. In addition to vitamins A and D, some of these animals were also provided with a vitamin E concentrate from wheat germ oil. Here also growth usually fell off after about 9 or 10 weeks and while increased doses of vitamins B_1 and B_2 temporarily stimulated growth, it could be effectively restored by a relatively small dose of dried brewer's yeast (Fig. 2). This did

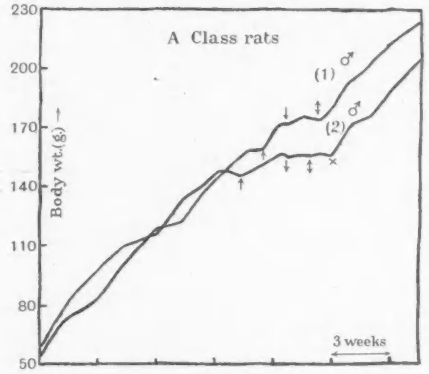

Fig. 2.

Curve (1). 0·5 cc. of fuller's earth preparation from wheat germ *plus* 1 cc. of 50 % alkalised marmite from the beginning of experiment; ↑ dose of each supplement doubled; ↓ dose of each quadrupled; ↕ 0·5 g. dried brewer's yeast daily.

Curve (2). 0·5 cc. of fuller's earth concentrate from wheat germ *plus* 1 cc. of 50 % alkalised marmite from the beginning of experiment; ↑ dose of each supplement doubled; ↓ 1 g. yeast residue in addition; ↕ 0·2 g. yeast ash in addition; × the above supplements replaced by 0·5 g. dried brewer's yeast.

not occur on feeding the ash of yeast or the residue of yeast obtained after extraction with boiling water. The supposition, that in these experiments yeast exerted its effect by means of its protein content, is rendered improbable by the fact that the same basal diet as described in Section A was used, which contained commercial "light white casein," a comparatively uninjured protein. The observations of Osborne and Mendel [1925], that rats required larger

amounts of "vitamin B" at later stages of the growing period, might receive a new interpretation in the light of these experiments.

C. *The effect of milk on the growth of rats receiving vitamin B_1 and B_2 preparations.*

It was found that when rats were exhibiting subnormal growth after being on vitamin B_1 and B_2 preparations for 9 or 10 weeks, 8–15 cc. of fresh unskimmed milk would produce a resumption in growth (Fig. 3). This appears

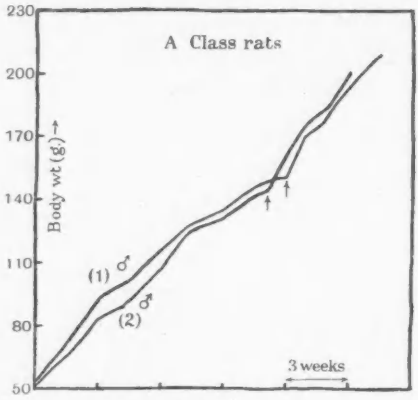

Fig. 3.

Curve (1). 0·5 cc. of fuller's earth preparation from wheat germ *plus* 2 cc. of 50 % alkalised marmite from the beginning of experiment; ↑ 8 cc. of unskimmed milk.

Curve (2). Same supplements as represented in Curve (1); ↑ 10 cc. of unskimmed milk.

to be far out of proportion to the amounts of vitamins B_1 and B_2 that milk contains, especially since, as has been shown in the previous sections, the effect of increased doses of vitamin B_1 and B_2 concentrates is not so marked. It would appear, therefore, that milk contains the additional factor present in yeast.

D. *Effect of milk on the growth of rats fed on a diet containing yeast.*

The observation that milk could supplement vitamins B_1 and B_2 led the writer to investigate what effect, if any, milk would have if the diet were already supplemented by yeast. Although in these early experiments, "light white casein" formed the protein of the basal diet, a distinct effect was observed if relatively large quantities of milk (15–25 cc.) were fed in addition. The experiments of Coward, Key and Morgan [1929] have since shown that "light white casein" contains some factor not present in "Glaxo vitamin-free casein." These findings had an obvious relation to those of the writer, and, therefore, in all subsequent experiments, "Glaxo vitamin-free casein" was substituted for the "light white" variety. It may, however, be stated here that "Glaxo casein," "light white casein" and purified caseinogen, as prepared by dis-

solving commercial caseinogen in dilute sodium hydroxide and precipitating by hydrochloric acid, show some difference in physical properties. While this question could not be investigated in detail for lack of time, it would appear that amongst these "light white casein" is most nearly present in the natural condition. It is probable that, for nutritional experiments involving the use of purified caseinogen, the original method of purification by Hammarsten, as adopted by Svedberg, Carpenter and Carpenter [1930] for the preparation of pure caseinogen for molecular weight determinations, would be the best. The use of ammonia instead of sodium hydroxide would probably lessen the risk of de-amination and racemisation.

In the following experiments B Class rats were used. The preliminary results were obtained with a basal diet composed of "Glaxo casein," commercial sucrose, yeast and salt mixture (Osborne and Mendel), without any added fat. In subsequent experiments, however, the following diet was used:

	%
Commercial sucrose	60
"Glaxo vitamin-free casein"	21
Palm kernel oil	8
Dried yeast	7
Salt mixture (Osborne and Mendel) ...	4

Daily doses of 2 drops of cod-liver oil were given separately to each rat in a dish, according to the usual procedure. However, in some of these experiments cod-liver oil (2·5 %) was incorporated in the diet with equally good results. In such a case, however, the diet should be made up every week. The palm kernel oil, which in some experiments was replaced by an equal amount of olive oil, was added in order to provide some fat in the diet [Burr and Burr, 1929]. On such a dietary the weight of the B Class male rats became steady or nearly so in the region of 200–220 g. after an experimental feeding period of 10–12 weeks. The corresponding weight of the female rats was 150–160 g. When the rats attained this stage, fresh "certified" milk in varying doses (10–25 cc.) had a pronounced effect, which could not be produced by (1) increasing the daily dose of cod-liver oil to 4 drops, (2) increased allowances of vitamin B_1 and B_2 preparations and yeast, (3) orange juice (5 cc. daily), (4) olive oil (6 drops daily), (5) lactalbumin (1 g. daily) in the form of "albulactin," (6) the addition of the following amino-acids to the diet in the stated proportions:

	%
Cystine	1
d-Lysine dihydrochloride	0·5
Tryptophan	1
Histidine monohydrochloride	1
Arginine hydrate	1
Tyrosine	1

Both wheat embryo oil (6 drops daily) obtained by extraction with cold ether, and wheat germ, heated at 130–170° for 3 hours—a treatment, which,

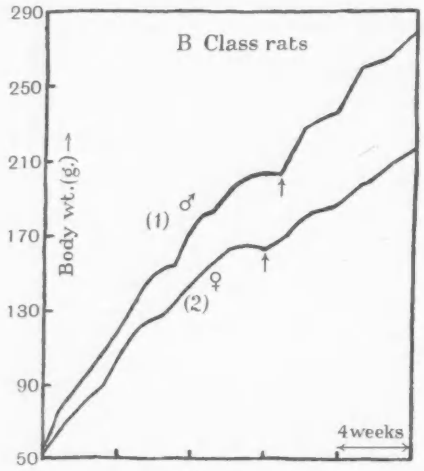

Fig. 4.

Curve (1). Basal diet as described in Section D; ↑ 15 cc. fresh certified milk daily.
Curve (2). Basal diet as described in Section D; ↑ 15 cc. fresh certified milk daily.

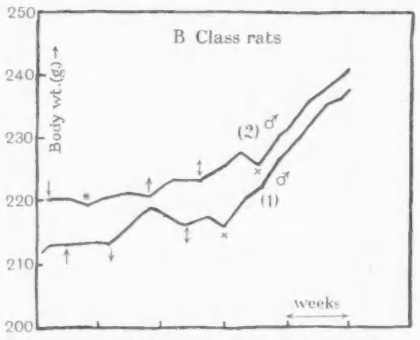

Fig. 5. Basal diet as described in Section D.

Curve (1). Body weight steady after 11 weeks' feeding on basal diet; ↑ 4 drops of cod-liver oil added daily; ↓ 0·5 g. dried yeast added daily; ↕ 5 cc. of orange juice daily; × 15 cc. fresh certified milk daily.

Curve (2). Body weight steady after 12 weeks' feeding on basal diet; ↓ 1 g. lactalbumin fed daily; ⊙ the mixture of essential amino-acids incorporated in the diet; ↑ 6 drops of olive oil daily; ↕ 15 % heated wheat germ incorporated in the diet; × 15 cc. fresh certified milk daily.

according to Evans and Burr [1927, 2], does not seriously affect vitamin E of the embryo—when introduced into the diet in a proportion of 15 % produced only a slight stimulation of growth. These experiments are illustrated

in Figs. 4 and 5. The effect of milk on the growth of males is more marked than on that of females. This difference in response between males and females has been noticed throughout this investigation and is probably to be correlated with the normally greater rate of growth of the males.

These experiments indicate that the factor present in milk cannot be identified with vitamin A, C, D, E or any of the factors present in yeast, and also that the deficiency in growth in the absence of this substance cannot be attributed to a deficiency of the so-called essential amino-acids investigated. The possibility cannot, however, be excluded that some other amino-acid or a peptide like that recently described by Dakin, West and Howe [1930], which the body may be unable to synthesise, may be the factor concerned.

E. *The sources of the new factor.*

The method by which this factor was assayed in different materials consisted in feeding these to the rats after their growth had stopped as the result of a feeding period of 10 weeks on the basal dietary described in the foregoing section. A weekly increase of 10 g. body weight for 3–4 weeks was considered as the standard of good growth. It has to be mentioned that even milk does not produce sustained growth for a very long time and the milk requirements also vary considerably from animal to animal. The results obtained with some materials are given in the following table.

Nature of material	Dose	Result
Egg-yolk	1–1·5 g.	Good growth
Egg-white	2 g.	Fairly good growth
Pig-liver	2 g.	Slight growth
Sheep-liver	2 g.	Slight growth
Spinach (dried at 35°) ...	1–1·5 g.	Good growth
Grass (dried at 35°) ...	1–1·5 g.	Good growth
Alfalfa	1–1·5 g.	Fairly good growth
Certified Grade "A" milk	10–25 cc.	Good growth

The fact that milk contains considerable quantities of this factor suggested the possibility of its extraction from "Glaxo" milk powder. But neither the ethereal extract of this powder (15 drops of the melted fat) nor the powder itself in daily doses of 1 g. was capable of producing growth. It is possible that the substance was injured in the drying process. The cream of milk was not much more effective than milk itself.

Both spinach and grass (dried at 35° by means of an electric fan) were extracted with ether for 6 hours in a Soxhlet in an atmosphere of nitrogen. But the extracts were almost entirely inactive, while the residues produced only slight growth.

Finally, it has to be mentioned that both "light white casein" and milk show very varying degrees of activity. Considering the possibility of seasonal variations a scrutiny was made of the records, but the evidence obtained was not conclusive. However, it appears possible that if the experiments were carried out under more standardised conditions with a fixed source of milk

supply, such a correlation might be revealed. Spinach and grass appear to be somewhat more active than milk, considering dry weights, and it seems plausible to suppose that the ultimate source of the milk factor is the food of the cow. A sample of carotene, kindly provided by Dr Moore, in a daily dose of 1 mg. gave negative results. Likewise, chlorophyll, when incorporated in the diet in the proportion of 0·15 %, proved entirely inactive.

F. *The stability of the factor present in milk to heat.*

The following table summarises the results, obtained very roughly.

Treatment	Time	Result
Boiling	40 mins.	Approx. half destroyed
Boiling	2½ hrs.	At least 75 % destroyed
Autoclaving at 12–13 lbs. pressure	15 mins.	Less than half destroyed
Autoclaving at 18 lbs. pressure	2½ hrs.	Wholly inactivated

G. *The question of food consumption.*

Very extensive food-intake records were kept, in some cases throughout the experimental period. These records show that, in many cases, when growth was resumed after administration of milk, the food consumption did not rise

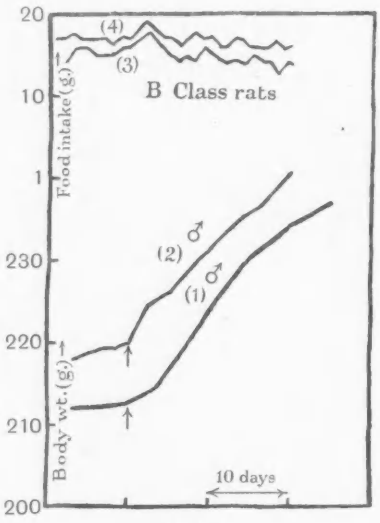

Fig. 6.

Curves (1) and (2). Basal diet as described in Section D; ↑ 15 cc. fresh certified milk daily.
Curve (3). Shows the food intake of the animal represented in Curve (1) before and after administration of milk.
Curve (4). Food-intake curve corresponding to Curve (2).

appreciably. Two such typical cases are shown in Fig. 6. Though in some other cases, the food intake rose, the fact, that it is possible to get resumption of

growth without greatly increased food consumption, suggests that this factor exerts its influence more directly on the assimilation of the food ingested than on appetite. If this is so, its effect should be reflected in a positive nitrogen balance.

Discussion.

It is not necessary to examine in detail all the papers referred to before. It is significant that all those investigators, working, sometimes, under considerably different conditions, observed the inadequacy of the known factors for the normal nutrition of the rat. In some of this work, however, it is not clear whether the limiting factor might not have been "vitamin B," while in some other cases specially "purified" proteins were used and the possibility could not be excluded that the effect of the supplement given was merely a good protein effect. Preparations of the third factor in yeast, called vitamin B_4, have recently been obtained by Reader [1930]. The experiments described in this paper have a great resemblance to those of Coward, Key and Morgan [1929]. They were led from a study of the relative qualities of "light white casein" and "Glaxo casein" in the basal diets of rats for vitamin A and B tests to the discovery that the former variety of caseinogen contained some factor, in which the latter variety was deficient. They subsequently traced this substance in milk and in many other materials. The writer's observations, however, started with milk. It appears probable that we are dealing here with the same substance. It should be stated, however, that Coward has found ox-liver to be rich in the factor, while, according to my observations, pig-liver and sheep-liver appear to be deficient in it. Coward and her collaborators have also observed that it can be extracted from wheat embryo by 90 % alcohol and by ether. My preliminary attempts to extract it from spinach and grass have so far failed. Attention should also be called to the work of Mason [1929], who independently showed that lettuce provided some additional factor for growth and, by a critical analysis, proved that this factor was not identical with vitamin E, which is present in large quantities in lettuce [Evans and Bishop, 1923].

The experiments described in this paper appear to eliminate the effect of all the known vitamins. That this factor cannot be identical with vitamin E is also shown by the fact that wheat germ heated under conditions in which vitamin E is not destroyed [Evans and Burr, 1927, 2] loses its activity. That untreated wheat germ is rich in this factor has been shown by Coward. The protein effect would appear to be excluded by the negative results obtained with lactalbumin and with the essential amino-acids tested. The idea that it might be some inorganic material seems to be similarly excluded by the observation that the factor in milk can be inactivated by autoclaving. That the deficiency described here cannot be of the type encountered by Burr and Burr [1929] is shown by the practically negative effect of olive oil. However, no evidence about its chemical nature has yet been obtained.

It appears that the factor present in yeast may also be present in milk, but the factor present in milk is not present in appreciable quantities in yeast. The two factors, therefore, seem to be different. It is possible that the observation of earlier workers that "complete" synthetic rations produce good growth was due to an insufficiently long observational period. It is also felt that the growth curves would to a very considerable extent depend on the dietary on which the stock had been reared and also on the strain of rats used.

Summary.

1. Accumulated evidence is presented to show that yeast contains a factor other than vitamin B_1 or B_2, and that milk contains a factor other than those present in yeast. Both the milk factor and the yeast factor are apparently required for the normal growth of rats. But even then entirely normal growth is not sustained.

2. The factor present in milk is different from other known vitamins, A, D, C or E.

3. The deficiency of the milk factor cannot be corrected by adding lactalbumin or the essential amino-acids to the diet.

4. Food-intake records show that increased growth on the administration of milk may occur without appreciably increased food intake.

5. The stability of the milk factor to heat has been investigated. The fact that it can be inactivated by autoclaving argues against the inorganic nature of this substance.

6. The factor present in milk is also present in egg-yolk, egg-white, spinach, grass and alfalfa, but much less so in pig- and sheep-liver.

7. Carotene or chlorophyll cannot replace the milk factor in the diet.

8. No satisfactory method has yet been devised for extracting the substance.

9. A method of assay of this factor is indicated.

10. Male rats show a much more marked response to this factor than females.

11. Milk and "light white casein" show appreciable variations in potency.

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